AREVERSE PHASE-HPLC/UV SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF ACEBROPHYLLINE AND MONTELUKAST SODIUM IN DOSAGE FORMS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

SUBMITTED

BY

KUMAR.NARAHARISETTI.S

(Reg. No. 261230953)

Under the guidance of

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DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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CERTIFICATE

This is to certify that the dissertation entitled**"A REVERSE PHASE-HPLC/UV SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF ACEBROPHYLLINE AND MONTELUKAST SODIUM IN DOSAGE FORMS"**submitted by **Kumar Narahari Setti.S**(Reg No:261230953) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam

Date:

Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,

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List of Abbreviations

UV	:	Ultra violet
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
g	:	Microgram
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
NMR	:	Nuclear Magnetic Resonance
Cm	:	Centimeter
Sec	:	Second
EMR	:	Electromagnetic Radiation
SD	:	Standard Deviation
RSD	:	Relative Standard Deviation
Nm	:	Nanometer
Monte	:	Montelukast sodium
Abr	:	Acebrophylline
API	:	Active Pharmaceutical Ingredient.

ABSTRACT

A simple, rapid, precise and highly selective Spectrophotometricmethod was developed for simultaneous estima tion of Montelukast sodium and Acebrophylline in tablet dosage form. This method, involves the measurement of absorbances of Montelukast sodium and Acebrophylline at the wavelengths of250nm and 273nm.Ethanol was used as solvent. Linearity was observed in the concentration range of 6-24µg/ml for Acebrophylline and 4-24µg/ml for Montelukast sodium.The accuracy of the method was confirmed by recovery studies of tablet dosage forms and was found to be 98.33% and 98.5%for Acebrophylline and Montelukast sodium respectively. Thus the proposed method was found to be rapid, specific, precise, accurate and cost effective quality control tool for the routine analysis of Montelukast sodium and Acebrophylline in bulk and combined dosage form. A selective, precise, isocratic and accurate stability indicating reverse phase high performance liquid chromatographic method was developed for the simultaneous determination of Montelukast sodium and Acebrophylline in the tablet dosage form. A chromatographic separation was achieved on reverse phase BDS Hypersil C18 column (250 ×4.6 mm, 5µ). The mobile phase consists of mixture of 10 mM phosphate buffer and Acetonitrile. The pH adjusted to 4 using 1% Orthophosphoric acid. The flow rate was 1ml/min and the effluents were monitored at the detection wavelength of 250nm. The retention times of Montelukast sodium and Acebrophylline were found to be 6.7 and 3.6 min respectively. The method was validated for the linearity, accuracy, precision,

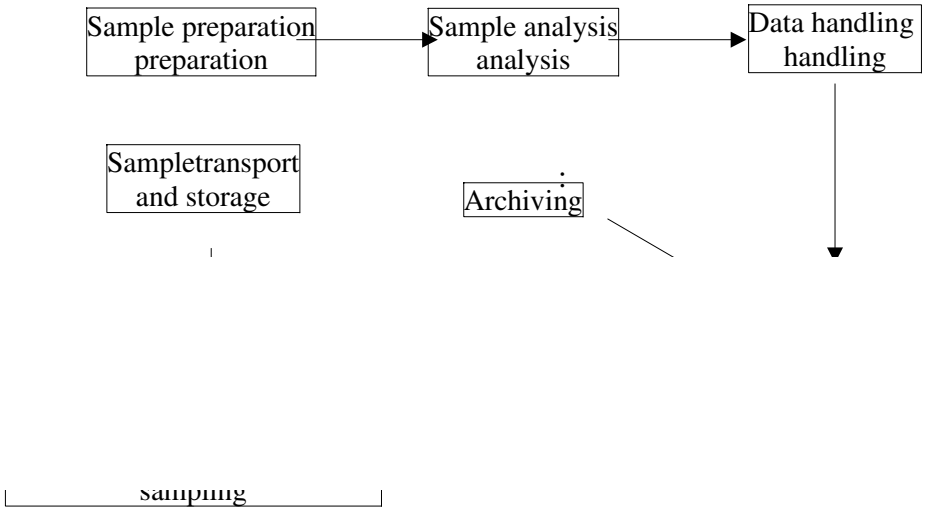
robustness, system suitability as per ICH guidelines. Montelukast sodium and Acebrophylline were found to be linear in the range of 100-1000 ng/ml with the recoveries of 99.8% and 102.0%.

1. INTRODUCTION

Pharmaceutical analysis is a branch of practical chemistry that involves a series of process for identification, determination, quantification and purification of a substance, separation of the components of a solution or mixture, or determination of structure of chemical compounds. The substance may be a single compound or a mixture of compounds and it may be in any of the dosage form. The substance used as pharmaceuticals are animals, plants, micro organisms, minerals and various synthetic products.

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials.

The major stages of an analytical process are described as follows:



Steps in analytical cycle

To be effective and efficient, analyzing samples requires expertise in:

1. The chemistry that can occur in a sample
2. The total amount of sample available
3. Concentration range of analyte
4. Analysis and sample handling methods for a wide variety of problems (the tools-of-the-trade)
5. Accuracy and precision of the method
6. Proper data analysis and record keeping

Analytical chemistry is the science of obtaining, processing and communicating information about the composition and structure of matter. In other words, it is the art and science of determining what matter is and how much it exists. Analytical chemistry also is concerned with developing the tools used to examine chemical compositions. It is concerned with the chemical characterization of matter both qualitatively and quantitatively.Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one or more of these components.

Analytical methods can be separated into classical and instrumental. Classical methods use separation techniques such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point. The quantitative analysis is achieved by measurement of weight or volume. Instrumental methods use an apparatus to measure physical qualities of the analyte such as lightabsorption, fluorescence, or conductivity. The separation of materials is accomplished by using chromatography or electrophoresis methods.

Common Techniques for analysis:

For analysis it is useful to consider chemical and physical characteristics that are useful for qualitative or quantitative analysis and thus analysis can be divided into:-

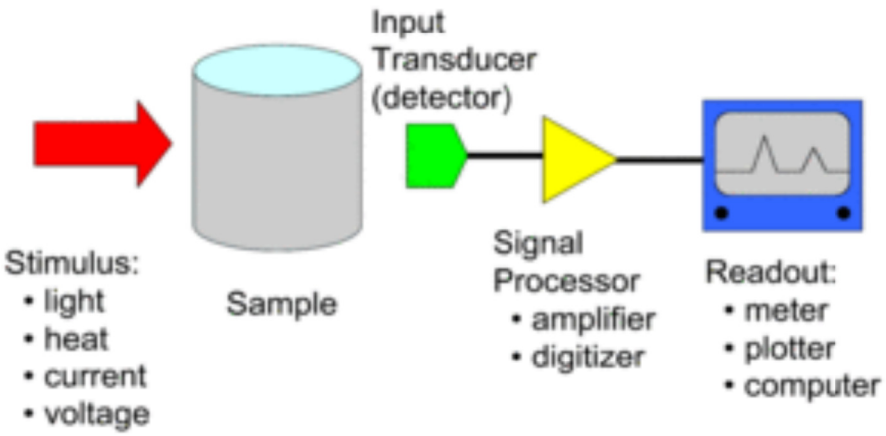
Qualitative analysis: It is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

Quantitative analysis: These techniques are mainly used to quantify any compound or substance in the sample. These techniques are based in (a) the quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained, (b) the characteristic movement of a substance through a defined medium under controlled conditions, (c) electrical measurement, (d) measurement of some spectroscopic properties of the compound.

The various physical properties employed for analysis with their instrumental methods are given in the table below.

S.no	Physical property measured	Instrumental method based on the measurement of property
1	Absorption of Radiation	Spectrophotometry (X-ray, UV, Visible, IR): Colorimetry, Atomic absorption, NMR.
2	Emission of Radiation	Emission spectroscopy(X-ray, UV, Visible, IR): Flame photometry, Fluorescence (X-ray, UV, Visible)
3	Scattering of Radiation	Turbidimetry, Nephelometry
4	Refraction of Radiation	Refractometry
5	Diffraction of Radiation	X-ray, electron Diffraction methods.
6	Rotation of Radiation	Polarimetry
7	Electrical Potential	Potentiometry
8	Electrical Conductance	Conductometry
9	Electrical Current	Polarography, Amperometric titrations
10	Mass-to-charge ratio	Mass spectrometry

Instrumental Analysis:



Block diagram of an analytical instrument showing the stimulus and measurement of response

1.1. UV-SPECTROPHOTOMETRY

Spectroscopyis the measurement and interpretation of electromagnetic radiation absorbed or emitted when the molecules or atoms or ions of a sample move from one energy state to another energy state.

Spectroscopy is a general methodologythat can be adapted in many ways to extract the information you need (energies of electronic, vibrational, rotational states, structure and symmetry of molecules, dynamic information).

Ultraviolet-Visible Spectrophotometry is one of the most frequently employed techniques in Pharmaceutical analysis. It involves the measurement of the amount of Ultraviolet (190-380nm) radiation by a substance in a solution.

A compound or drug which posses conjugated double bond absorbs UV radiation at a specific wavelength and this character of the drug is specific for a fixed solvent system. The wavelength at which maximum absorption occurs is called λmax.

It is independent of concentration. For a drug to be measured by the ultraviolet analytical method, it should follow the Beer's-Lambert's law. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Beer-Lambert Law

The Beer-Lambert Law states the amount of intensity of light when passing through any sample decreases exponentially with increase in thickness of the sample and the concentration of the medium.

Lambert Beer's law is a mathematical means of expressing how light is absorbed by matter.

This relationship may be expressed as:

$$A = \varepsilon dc$$

A= Absorbance

ε = molar extinction coefficient

d = Path length in cm

c = molar concentration

1.1.1. Quantitative Spectrophotometric assay

Assay of single component sample

a) Single standard (or) Double-point standardization

It involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The concentration of the substance in the sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$C_{test} = \frac{A_{test} \times C_{std}}{A_{std}}$$

C_{std} = Concentration of the standard solution
C_{test} = Concentration of the sample
A_{test} and A_{std} = Absorbances of the sample and standard solutions.

b) Calibration graph method

In this procedure the absorbances of a number of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution.

Assay of Multi-Component samples:

The assay of components of mixture sample can be done by following methods:

1) Simultaneous Equation method
2) Absorbance Ratio method
3) Geometric Correction method
4) Difference spectrophotometry
5) Derivative spectrophotometry

The basis of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths,

a) The absorbance of a solution is the sum of absorbances f the individual components or

b) The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

Simultaneous Equation method

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λmax of the other, it may be possible to determine both drugs by the technique of simultaneous equation.

The information required is:

a) The absorptivities of X at λ_1 and λ_2, ax_1 and ax_2 respectively.
b) The absorptivities of Y at λ_1 and λ_2, ay_1 and ay_2 respectively.
c) The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.
d) Let C_x and C_y be the concentrations of X and Y respectively in the diluted sample.

Two equation are constructed based upon the fact that the sum of individual absorbance of X and Y.

At λ_1, $A_1 = ax_1bc_x + ay_1bc_y$

At λ_2, $A_2 = ax_2bc_x + ay_2bc_y$

$$C_x = \frac{A_2ay_1 - A_1ay_2}{ax_2ay_1 - ax_1ay_2}$$

$$C_y = \frac{A_1ax_2 - A_2ax_1}{ax_2ay_1 - ax_1ay_2}$$

Using the above equations, concentrations of individual components in a mixture can be determined.

Difference spectrophotometry

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by the technique of difference spectrophotometry.

The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different spectral characteristics.

The criteria for applying spectrophotometry to the assay of a substance in the presence of other absorbing substances are that:-

a) Reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents.
b) The absorbance of the interfering substances is not altered by the reagents.

Derivative Spectrophotometry

Derivative Spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by the reference to a Gaussian band which represents an ideal absorption band.

The first derivative (D^1) spectrum is a plot of the rate of change of absorbance with wavelength against wavelength i.e. a plot of the slope of fundamental spectrum against wavelength or a plot of $\delta A\ \delta\lambda_{Vs}\ \lambda$.

The second derivative (D^2) spectrum is a plot of the curvature of the D^0 (Zero order spectrum) against wavelength or a plot of $\delta^2 A\ \delta^2\lambda_{Vs}\ \lambda$.

The spectral transformations confer two principle advantages on derivative spectrophotometry.

1) An even order spectrum is of narrow spectral bandwidth than its fundamental spectrum.
2) Derivative spectrophotometry discriminates in favour of substance of narrow spectral bandwidth against broad bandwidth substances.

Hence substance of narrow spectral bandwidth displays larger derivatives, amplitudes than those of broad bandwidth substances.

Absorbance Ratio Method

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that, for a substance which obeys Beers law at all

wavelength, the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length.

Here absorbances are measured at two wavelengths one being the λ of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), i.e. an iso-absorptive point.

1.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography is the separation of a mixture into individual components using a stationary and mobile phase. Early liquid chromatography was carried out in glass columns with diameters of 1 to 5 cm and lengths of 50 to 500 cm. The average diameter of the solid stationary phase particles was usually in the 100 to 200 micron range. Recent technology has allowed for the development of packing material with relatively small particle size diameter (3-10 micron). This technology resulted in the development of columns with very high efficiencies, and consequently has involved the use of more sophisticated instrumentation to perform at increased pressure and flows; Hence the term High Performance Liquid chromatography (HPLC).

The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high-pressure pump. Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it

incorporates a wide choice of detection methods. With the use of Post-column derivatization methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific tools.

1.2.1. Parameters used in Chromatographic Characterization

Retention: The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of this are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$K_A = \frac{V_A V_0}{V_0} = \frac{t_A - t_0}{t_0}$$

Where,

V_A = Elution volume of A

V_0 = Elution volume of a non retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes.

Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times is better when data need to be transferred between different chromatographs.

Resolution: The distance between any two adjacent peaks in a multi peak chromatogram is referred to as Resolution 'R_s' and is calculated as

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

Where,

t_1 and t_2 are the retention times for the latest and the earliest eluting peak and W_1 and W_2, are the peak width at baseline.

$R \geq 1$ = Components completely separated

$R \leq 1$ = Components overlap.

Capacity Factor (K^1) - The retention of the analyte expressed as the number of void volumes of the system, needed for the peak to elute is called the capacity factor. The expression for K^1 is

$$K^1 = \frac{t_r - t_0}{t_0}$$

Where,

t_r = retention time

t_0 = void volume

Theoretical plates (N)

The number of theoretical plates generated on a column is a measure of its performance. The definition of N is

$$N = 16\left(\frac{tr}{W}\right)^2$$

Where,

t_r = retention time

W = peak width

'N' may also be calculated from the width along the baseline of the peak.

This is accomplished by extending tangents from the two peak inflection points through the baseline.

Separation factor (□)

This parameter is used to quantify the separation between adjacent peaks. Ideally, the peaks should not overlap, that is they should be baseline resolved. This condition is meant for peaks of similar size when □ > 1.15. The separation factor is calculated as follows.

$$\square = \frac{K^1_2}{K^1_1}$$

Where,

The subscripts refer to the order of elution. □ is always ≥1.

Asymmetry – The asymmetry is a tool for quickly determining how much if any, of an eluting peak profile deviates in shape from a normal distribution. The subscripts 'X' refer to the percentage of peak height at which the asymmetry is determined.

Eg: A^{10} (determined at 10% peak height)

The equation for determining peak asymmetry is

$$A_X = b/a,$$

Where,

'b' = The distance between the perpendicular connecting the baseline to peak maximum and the latest eluting portion of the curve.

'a' = The distance between the perpendicular connecting the baseline to the peak maximum and the earliest eluting portion of the curve.

1.4. METHOD VALIDATION

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated,

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.
- Whenever the method is changed, and the change is outside the original scope of the method.

The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics.

The parameters as defined by the ICH and by other organizations

- Specificity
- Selectivity
- Precision

Repeatability

- Intermediate precision
- Reproducibility

- Accuracy
- Linearity
- Range
- Limit of detection
- Limit of quantitation
- Robustness
- Ruggedness

SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

A specific method can accurately measure the analyte of interest even in the presence of potential sample components (placebo ingredients, impurities, degradation products etc.) When criteria for specificity are not met, this often indicates that the method is not sufficiently developed; furthermore, it is likely that criteria for accuracy, precision, and linearity may also not be fulfilled. A major objective of determining specificity is to ensure "peak purity" of the main compound to be determined, in other words, confirm that no related compound or product ingredient co elutes and interferes with the measurement of the assayed compound.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels:

- Repeatability

- Intermediate precision
- Reproducibility

1) Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time.

Repeatability of a method can be determined by multiple replicate preparations of the same sample. This can be done either by multiple sample preparation (n = 6) in the same experiment or by preparing 3 replicates at 3 different concentrations.

2) Intermediate Precision

Intermediate precision expresses within laboratories variations: Different days, different analysts, different equipments etc.

3) Reproducibility

Reproducibility expresses the precision between laboratories. This is optional validation parameter that requires demonstration of laboratory - to - laboratory variation only if multiple laboratories use the same procedure.

ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Accuracy is often determined by recovery studies in which the analytes are spiked into a solution containing the matrix. The matrix (placebo in formulations)

should be found not to interfere with the assay of the compound(s) of interest.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample.

Different Approaches for linearity determination.

ii) The first approach is to weigh different amounts of authentic sample directly to prepare linearity solutions of different concentrations.

ii) Another approach is to prepare a stock solution of high concentration, then perform serial dilution from the stock solution to obtain solutions of lower concentrations for linearity determination.

RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of the analyte in the sample (including these concentrations) for which it has been shown that the analytical procedure has a suitable level of precision, accuracy and linearity.

LOD AND LOQ

Detection limit (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. In chromatography detection limit is the injected amount that results in a peak with a height at least twice as high or thrice as high as baseline noise level.

S/N = 2/1 or 3/1

Quantitation limit (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. In chromatography, quantitation limit is the injected amount that results in a peak with a height ten times as high as baseline noise level.

S/N = 10/1

Different approaches to determine LOD and LOQ

1. Signal to noise approach

$$\textit{Quatitation limit at each concentration} = 10\,x\frac{Concentration\,(in\ \ related\ substance)}{S/N(average\ \ at\ \ each\ \ concentration)}$$

2. Standard deviation approach

$$QL = 10 \times \frac{SD}{S} \qquad DL = 3.3 \times \frac{SD}{S}$$

SD = Standard deviation of the response near QL

S = Slope of the linearity curve near QL

ROBUSTNESS

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations, in method parameters and provide an indication of its reliability during normal usage. This is to verify that the method performance is not affected by typical changes in normal experiments. Therefore, the variation in method conditions for robustness should be small and reflect

typical day to day variation.

RUGGEDNESS

The United States Pharmacopoeia (USP) define ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.

2. NEED FOR THE STUDY

According to WHO, a drug may be defined as any substance or product that is used or intended to be used for modifying or exploring physiochemical systems or pathophysiological stages for the benefit of the patient. Pharmaceutical Chemistry is a science that makes use of general laws of chemistry to study drugs i.e., their preparation, chemical natures, composition, structure, influence on an organism. It also studies the physical and chemical properties of drugs, the methods of quality control and the conditions of their storage etc. The family of drugs may be broadly classified as

1. Pharmacodynamic agents and

2. Chemotherapeutic agents

We use drugs almost every single day. The quality of the drug is the most essential feature and that parameter directly affects the life of the customer. The quality of any product or material can be judged by only analysing it. It is necessary to find the content of each drug either in bulk or single or combined dosage forms for purity testing. It is also essential to know the concentration of the drug and it's metabolites in biological fluids after taking the dosage form for treatment. The scope of developing and validating an analytical method is to ensure a procedure which is specific and accurate for a particular analyte. The primary objective is to improve the conditions and parameters to be followed in the method development and validation.

The review of literature revealed that several methods are available for the determination of Acebrophylline and Montelukast sodium individually. Reported method for estimation Acebrophylline in dosage form are spectrophotometry, RP-HPLC, and HPTLC and similarly for estimation of Montelukast sodium in dosage form are spectrophotometry, spectrofluorimetry, Voltametric, RP-HPLC and HPTLC.

But, there is no any analytical method has been reported yet for combination of these drugs. There for the present research work aims to develop a simple, sensitive, accurate and reproducible method for simultaneous estimation of Acebrophylline and Montelukast sodium in combined dosage form by spectrophotometric method

2.2 OBJECTIVES

Following are the objectives of present work

- To perform method development for the drugs
- To study effect of various mobile phases used in method development
- To determine the drug content in given pharmaceutical dosage form

- To validate analytical methods as per ICH guidelines
- To demonstrate that it is suitable for its intended purpose
- To establish identity, detect and quantitated impurities and to assess characteristics

Following parameters according to ICH Guidelines to be validated

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantification
- Robustness

3.LITERATURE REVIEW

1. Bhagavati Solanki Bhagavati Solanki, Harshal Trivedi, Ankita Khodiyar, Falguni Tandel, Lalit Lata Jha, Rajesh KS [13], developed Derivative Spectroscopic Method For Estimation of Acebrophylline In Bulk And Its Dosage Form & In Presence of Impurity, Ambroxol HCl. A sensitive, selective and validated first derivative spectrophotometric method using methanol have been proposed for the determination of Acebrophylline which is widely used as anti asthmatic agent. The method is accurate, precise and linear over a range of 5-50µg/ml for Acebrophylline. The limit of detection was observed as 0.178µg /ml, the limit of

quantification was observed as 0.598µg/ml. The %RSD is less than 2% in methanol. Method accurately estimates Acebrophylline in presence of Ambroxol with 99-100% recovery. The method can be applied for Acebrophylline tablet formulation with 99-100% recovery.

2. Sunil R Dhaneshwar, Vaijanath N. Jagtap [14], had developed stability-indicating RP-HPLC-PDA method and subsequently validated for the determination of Acebrophylline in commercial capsules. The proposed HPLC method utilizes waters Cosmogel C18 column (150 mm x 4.6 mm, 5.0 µ particle size) and mobile phase consisting of methanol:acetate buffer (20:80 v/v) pH adjusted to 6 with glacial acetic acid at a flow rate of 0.85 mL/min and column was maintained at 500 C. Quantitation was achieved with UV detection at 274 nm based on peak area with linear calibration curves at concentration ranges 0.5-200 µg/mL for ACB (R2 > 0.999). ACB and its drug products were exposed to acid, base and neutral hydrolysis, oxidation, heat and photolytic stress conditions. Under all these conditions, degraded products were well separated. The method was validated according to the ICH guidelines with respect to accuracy, precision, linearity, specificity, limits of detection, limits of Quantitation and robustness.

3. Uday D Thesia et al[15] developed First order, Area under curve and dual wavelength Spectrophotometric methods for simultaneous estimation of Montelukast sodium and Acebrophylline in bulk drug and in pharmaceutical formulation. All these methods utilizes methanol as a solvent. In First order spectroscopy 236.4nm and 334.4nm were selected as ZCP (zero crossing point) for determination of both the drugs simultaneously. In Area Under Curve the wavelength ranges of 277-287 nm and 270-280 nm were selected as analytical

wavelength ranges for the determination of area of both the drugs in pure and in combined formulation. In Dual wavelength method, the absorbance difference was determined within 244nm and 254nm for the the determination of Montelukast sodium, and absorbance difference within 265nm and 285nm for the determination of Acebrophylline. The results of analysis of all methods have been validated statistically. Lower value of % RSD indicates that these methods were simple, rapid and accurate, hence can be used for routine simultaneous estimation of these drugs in formulations.

4. Ravisanker M, Uthirapathy S et al[16], developed and validated RP-HPLC method for simultaneous estimation of Fexofenadine hydrochloride and Montelukast sodium in bulk drug and marketed formulation, using water symmetry C8 column. The mobile phase consisted of 0.05M potassium dihydrogen orthophosphate and Acetonitrile in the ratio of 35:65% v/v (pH

   6 with TEA). The flow rate was kept at 1.0ml/min and the wavelength selected for the Quantitation was 226 nm. The retention time was found to be 2.127 min for Fexofenadine and 5.650 min for Montelukast sodium. The linearity was found to be in the range of 4.8-28.8 µg/ml and 0.4-2.4 µg /ml for Fexofenadine and Montelukast respectively with the correlation coefficient of 0.999. The mean recoveries for Fexofenadine and Montelukast were observed as 99.85% and 100.19% respectively, and relative standard deviation was less than 2%. The assay value for Fexofenadine and Montelukast were found to be 100.55% and 100.40% respectively.

5. Konam K, Hariprasad P, Lukaraju PS, Sirajudeen MA, Fareedullah MD, Ahmed I [17] had developed and validated high-performance thin-layer chromatographic method for the estimation of Montelukast sodium and Levocetirizine

hydrochloride simultaneously in combined dosage form. In this method stationary phase used was precoated silica gel 60F 254 and mobile phase used was a mixture of Chloroform: Benzene: Methanol: Toluene (5:7.2:1:0.2). The detection of spots was carried out at 286nm. The calibration curve was found to be linear between 500-1500ng/spot for Montelukast sodium and 1000-5000ng/spot for Levocetirizine hydrochloride.

6. Kumar BVVS, Mathur P, Rajesh N, Rao ND, Satyanarayana P [18] had developed and validated reversed phase HPLC method for the determination of Levocetirizine hydrochloride and Montelukast sodium in pharmaceutical dosage forms using C8 column [4.6 x 150mm, 3.5mm, Make: XTerra] with a mobile phase of potassium di hydrogen

   orthophosphate buffer and acetonitrile (60:40 v/v) at a flow rate of 0.8ml/min and detection was carried out at 230nm. The retention time of Levocetirizine hydrochloride and Montelukast sodium was found to be 2.432 min and 6.218 min respectively. The method was observed to be linear in the concentration range of 30-70µg/ml for both the drugs. The LOD values were found to be 3.36ng/ml and 3.20ng/ml and the LOQ were 9.90ng/ml and 9.86ng/ml for Levocetirizine hydrochloride and Montelukast sodium respectively. The mean recoveries were found to be 100.2% and 99.7% for Levocetirizine dihydrochloride and Montelukast sodium, respectively.

7. Patel NK, Pancholi SS [19] had developed spectrophotometric method for Montelukast sodium and Levocetirizine dihydrochloride in tablet dosage form by AUC curve method. The area under curve was selected from 263.6nm to 293.6nm and 222nm to 242nm for determination of Montelukast sodium and Levocetirizine dihydrochloride respectively. Both drugs showed linearity in the

concentration range of 5-30µg/ml. The limit of detection (LOD) and limit of quantification (LOQ) were determined by visual methods as suggested in ICH guidelines, which were found to be 1.6µg/ml and 4.8µg/ml at 222-242nm, 1.06µg/ml and 3.1µg/ml at 263.6-293.6nm for Montelukast sodium respectively. The LOD and LOQ were 1.23µg/ml and 3.71µg/ml at 222-242nm for Levocetirizine dihydrochloride respectively. The % assay results were found to be 98.90 ± 0.61% for Montelukast sodium and 98.75 ± 0.96% for Levocetirizine dihydrochloride of the labeled claim. The result of recovery studies were found to be in the range of 97.87-98.95% and 98.63-99.68% for Montelukast sodium and Levocetirizine dihydrochloride, respectively.

8. Choudhari V, Kale A, Abnawe A, Kuchekar B, Gawli A, Patil N[20] had developed ratio derivative spectrophotometric method for the simultaneous determination of Montelukast sodium and Levocetirizine dihydrochloride in pharmaceutical preparations. This method involved measurement of first derivative amplitude of ratio spectra at 250.4nm for Montelukast sodium and 238.4nm for Levocetirizine dihydrochloride as two wavelengths for estimation. Beer's law was obeyed in the concentration range of 4-12µg/ml and 2-6µg/ml for Montelukast sodium and Levocetirizine dihydrochloride respectively. LOD values were found to be 0.09µg/ml and 0.178µg/ml and the LOQ values are found to be 0.277µg/ml and 0.591µg/ml for Montelukast sodium and Levocetirizine dihydrochloride respectively. The % assay was found to be 96.86% for Montelukast sodium and 99.63% for Levocetirizine dihydrochloride. Results of recovery studies were found to be in the range of 99.79%-100.68% with % RSD values ranging from 0.394%-0.777% for Montelukast sodium and

99.44% to 100.2%, with % RSD values ranging from 0.425% to 0.808% for Levocetirizine dihydrochloride.

9. Alsarra I, AI-Omer M, Gadkariem EA and Belal F [21], studied voltametric determination of montelukast sodium in pharmaceutical dosage form and human plasma. The voltametric behavior of Montelukast was observed using Cyclic voltametry Direct current (DCt), Differential Pulse Polarography (DPP) and Alternating Current (ACt) polarography. Montelukast exhibited well defend cathodic waves over the pH range of 1-5. No anodic waves were produced over the same pH range at pH 1, the

analytical pH: the diffusion current constant (Id) was 2.2 ± 0.01μAI/ mol. The current concentration plot was found rectilinear over the range 2-20μg/ml with correlation coefficient (n=10) of 0.9943. The lower limit of detection (S/N= 2) was found at 0.2μg/ml (3.41×10^{-7}M) .The wave had characterized as being diffusion controlled. Although adsorption phenomenon played a limited role in the electrode reaction.

10. Radhakrishna T, Narsaraju A, Ramakrishna M, Satyanarayna A[22] developed and validated HPLC and second order derivative spectrophotometric method for simultaneous estimation of Montelukast and Loratadine from pharmaceutical formulation. HPLC separation was achieved with a symmetry C18 column and sodium phosphate buffer (pH adjusted to 3.7): Acetonitrile (20:80 %v/v) as elute, at a flow rate of 1.0 ml/min and UV detection was performed at 225nm. 5-Methyl 2-nitrophenol was used as an internal standard. In the second order derivative spectrophotometry , for the determination of Loratadine the zero crossing technique was applied at 276.1

nm, and for Montelukast peak amplitude at 359.7 nm (tangent method) was used. The percentage recovery was found in the range of 99.05-100.47 for Montelukast and 99.00-100.02 for Loratadine by HPLC method and 98.43-101.62 for Montelukast and 98.41-100.98% for Loratadine by second order derivative method.

11. J Maniya, H Raj, K Muralikrishna, P Lakshmi, B Khanpara, V Shelar [23] developed and validated reversed phase high-performance liquid chromatographic method. The method was performed on Unisphere C18 column (250mm X 4.6 mm i.d., 5 µm particle size) at 40oC; the mobile phase, consisting of a mixture of Sodium acetate (200mM, pH adjusted to 6 with glacial acetic acid) : Methanol : Acetonitrile (20:30:50 v/v/v) was delivered at a flow rate of 1.0mL/min and detector wavelength at 272 nm. The retention time of Theophylline-7-acetate and Ambroxol was found to be 2.22 and 4.52 min respectively as an individual component of Acebrophylline and 5.22 min for Montelukast sodium. Linearity ranges were 100-600 and 5-30 µg/ ml with limit of detection values of 0.52 and 0.05µg/ml for Acebrophylline and Montelukast sodium respectively. In First order derivative spectroscopy method, absorbance of Acebrophylline was measured at 282 nm (Zero Crossing Point of Montelukast sodium) and absorbance of Montelukast sodium was measured at 368 nm (Zero Crossing Point of AcebrophyllineResults of assay and recovery studies were statistically evaluated for its accuracy and precision. Correlation coefficients (r2) of the regression equations were greater than 0.999 in all cases

4.AIM AND OBJECTIVE OF WORK

2.1 AIM OF WORK:

The scope of developing and validating a method is to ensure a suitable strategy for a particular analyte which is more stable ,cheap specific accurate precise, and less time consumingfor the simultaneous estimation of Acebrophylline and Montelukast in

tablet dosage form and validate as per ICH guidelines. The existing available literature reveals that there are no available analytical methods for simultaneous estimation of Acebrophylline and Montelukast.

Hence attempt is being made to develop sensitive, simple, accurate and robust RP-HPLC method for simultaneous estimation of Acebrophylline and Montelukast in bulk and tablet dosage form. And to validate the developed method by establishing the parameters like accuracy, precision, linearity, LOD, LOQ, robustness.

The plan of work is as follows:

i) Study of physicochemical properties of drug, (pH, pKa, solubility and molecular weight)

ii) Preparation of drug standard and sample,

iii) Simultaneous estimation of Acebrophylline and Montelukast sodium from formulation by UV spectrophotometric method using Simultaneous Equation Method

iv) Optimization chromatographic conditions like,

   a. Selection of wavelength

   b. Selection of initial separation conditions

   c. Nature of stationary phase

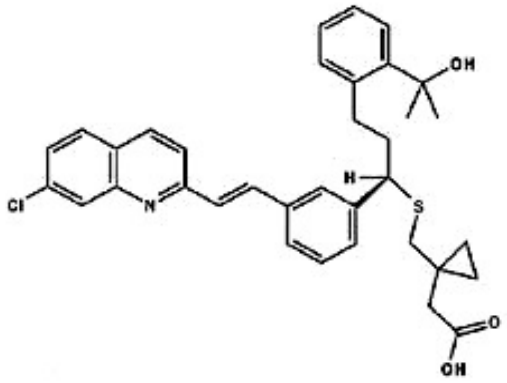
   d. Nature of mobile phase (pH, solvent strength, solvent ratio and flow rate)

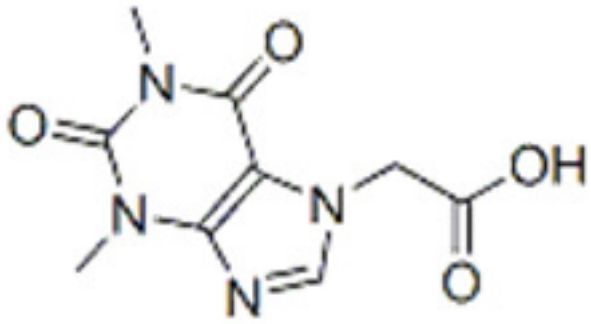
v) Study of system suitability parameters,

vi) Validation of proposed method by RP-HPLC.

vii) Applying developed method to marketed formulation.

5. DRUG PROFILE

MONTELUKAST	
Structure	
Molecular formula	$C_{35}H_{36}ClNO_3S$
Molecular weight	586.183
Chemical IUPAC Name	2-[1-({[(1R)-1-{03-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl}-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanyl}methyl)cyclopropyl]acetic acid
Solubility	Freely soluble in ethanol, methanol and water Insoluble in acetonitrile.
Category	• Anti-Asthmatic Agents • Antiarrhythmic Agents • Leukotriene Antagonists
Class	• Phenylpropenes • Cumenes and Derivatives • (Iso)quinolines and Derivatives
Available dosage form	Tablets

ACEBROPHYLLINE	
Structure	
Molecular formula	$C_{22}H_{28}Br_2N_6O_5$
Molecular weight	616.30
Chemical IUPAC Name	1,2,3,6-Tetrahydro-1,3-dimethyl-2,6-dioxo-7H-purine-7-acetic acid with trans-4-[[(2-Amino-3,5-dibromophenyl)methyl]amino]cyclohexanol
Solubility	Freely soluble in water, ethanol. Insoluble in diethyl ether, Acetonitrile.
Category	Mucolytic – Bronchodilator
Class	Anthocyanidins. (Iso)flavones and Derivatives
Available dosage form	Tablet.
Brand Name	Surfolase

Brand name of Acebrophylline and Montelukast sodium Hydrochloride:

ABROFYL-M (Manufacturer**: Alkem laboratories ,(Mumbai)**

Drug	Dose
Acebrophylline	200mg
Montelukast sodium Hydrochloride	10mg

Combination of Acebrophylline and Montelukast sodium hydrochloride

Acebrophylline and Montelukast sodium Hydrochloride is available in combined dosage forms as film coated tablets (**ABROFYL-M**). Each tablet contains 200mg of Acebrophylline and 100 mg of Montelukast sodium Hydrochloride. It is used for the treatment of Respiratory Tract Infection. Acebrophylline (ACBR) , 1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7H-Purine-7-aceticacid compd. with trans-4-[[(2-amino-3,5-dibromophenyl)methyl]amino]cyclohexanol is the salt obtained by reaction of equimolar amounts of theophylline-7-acetic acid, a xanthine derivative with specific bronchodilator activity and ambroxol, a Mucolytic and expectorant. Montelukast sodium (MTKT), [R-(E)]-1 -[[[1-[3-[2-(7-chloro 2quinolinyl) ethenyl] phenyl]-3-[2-(1-hydroxy-1 methylethyl) phenyl] propyl] thio] methyl] cyclopropane acetic acid, monosodium salt is a selective and orally active leukotriene receptor antagonist that inhibits the cysteinyl leukotriene CysLT1 receptor. By blocking Leukotrienes, improves asthma symptoms, help to control asthma and improves seasonal allergy symptoms. The combination of these two drugs are available in the market for the treatment of chronic obstructive pulmonary disease (COPD) and bronchial asthma and also clinical trials has been reported for this combination in India.

6. MATERIALS AND INSTRUMENTS

a) Pure drug samples

Both the drug samples of Acebrophylline and Montelukast Sodium were received as a gift samples from AMIS Pharmaceuticals -Vadodhara and Dr. MACS BIO PHARMA., Hyderabad respectively.

b) Chemicals and solvents used

- Methanol**:** HPLC grade, Qualigens Fine Chemicals, Mumbai.
- HPLC water: Qualigens Fine Chemicals, Mumbai.
- Orthophosphoric acid :88% GR, Merck, Mumbai.
- Triethylamine: S.D. Fine Chemicals Ltd., Mumbai.

c) Instruments

- Schimadzu Digital Electronic Balance- BL 220H.
- Lab India SAB 5000, pH meter.
- Value Vacuum pump.
- Schimadzu UV-1800, UV/Vis-Spectrophotometer.
- Schimadzu HPLC, BDS Hypersil C18 column, UV detector.
- Ultrasonic cleaner, Life care equipmentspvt.ltd.

Fig: 6.1. UV-Visible Spectrophotometer

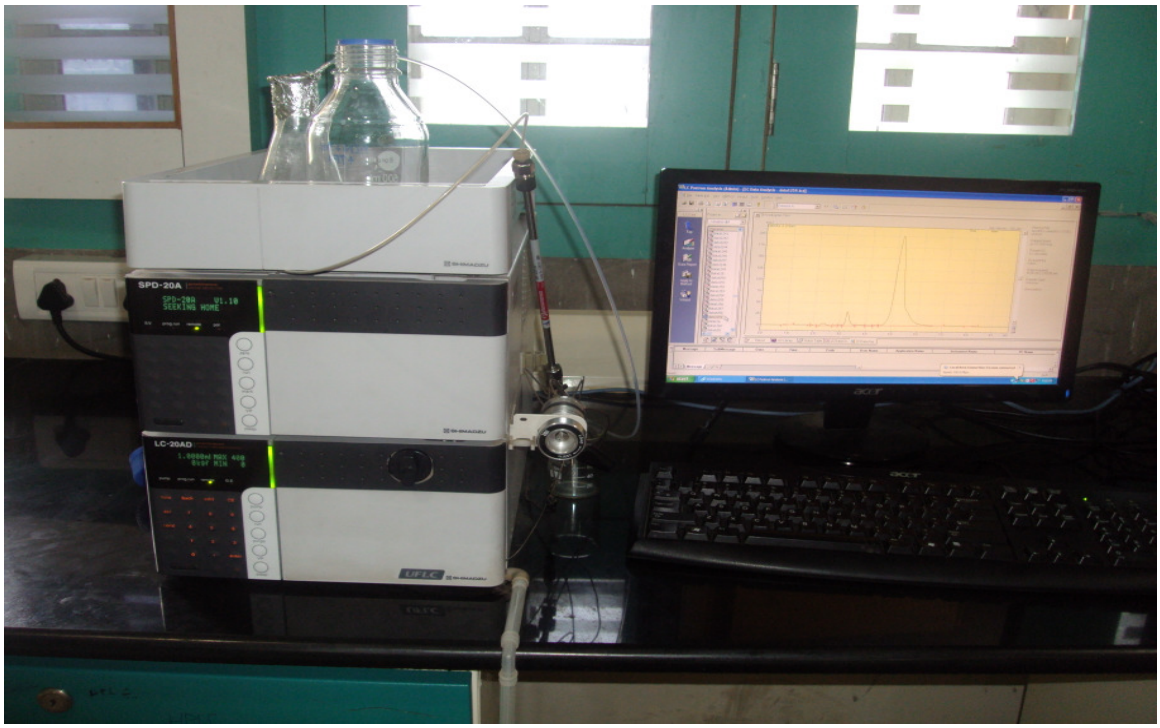


Fig: 6.2. High Performance Liquid Chromatography (HPLC)

Fig: 6.3. pH Meter

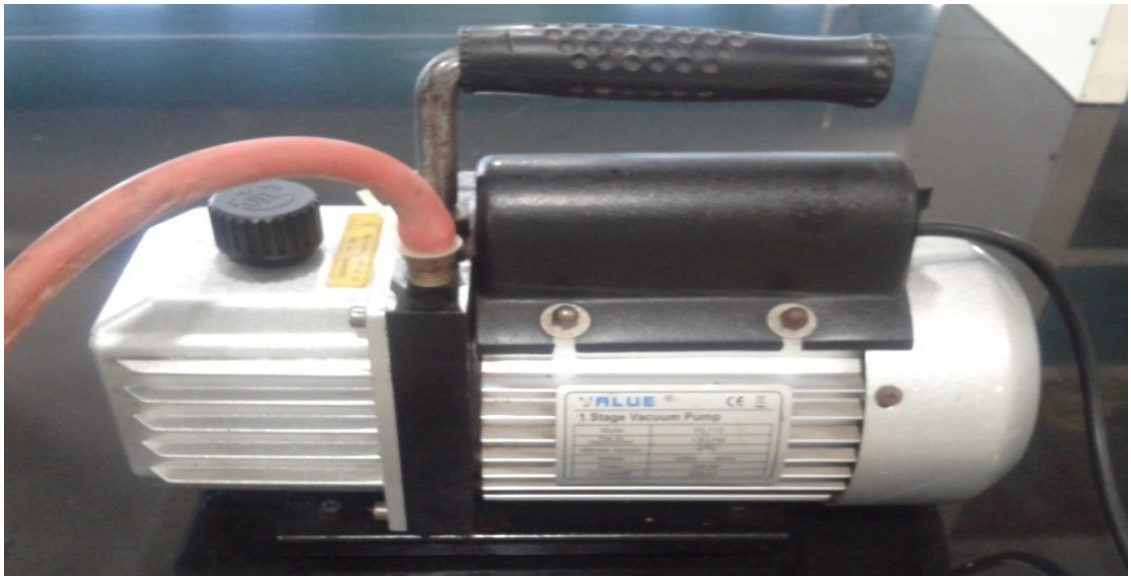


Fig: 6.4. Value 1 stage, Vacuum pump

Fig: 6.5. Shimadzu Digital Electronic Balance – BL 220H

Fig: 6.6. Fast clean Ultrasonicator

7. METHODOLOGY

7 .1 DEVELOPMENT AND VALIDATION OF UV SPECTROSCOPIC METHODFOR SIMULTANEOUS ESTIMATION OF ACEBROPHYLLINE AND MONTELUKAST SODIUMIN COMBINED DOSAGE FORMS BY SIMULTANEOUS EQUATION METHOD

7.1.1. Selection of solvent

Solutions of Acebrophylline and Montelukast Sodium were prepared in different solvents like methanol, ethanol, acetonitrile and UV spectrum of each were recorded by scanning between 200-400 nm.

An overlain spectrum of Acebrophylline and Montelukast were prepared in different solvents like methanol, ethanol etc.,. Better absorbances were observed for both the drugs when ethanol is used as a solvent as shown in the figure 7.11. Hence, ethanol was selected as solvent for present study.

From the spectra of drugs in ethanol, four trial wavelengths were selected (250, 273,296 and 315nm) for their simultaneous estimation. Different concentrations of Acebrophylline(6 to 24µg/ml), Montelukast (6 to 24µg/ml) and mixture of Acebrophylline and Montelukast were prepared, scanned and absorbances were noted at these three wavelengths.From these data, it was noted that, at the wavelengths 250 and 273 nm, good linearity was observed and hence these wavelengths were fixed for the study.

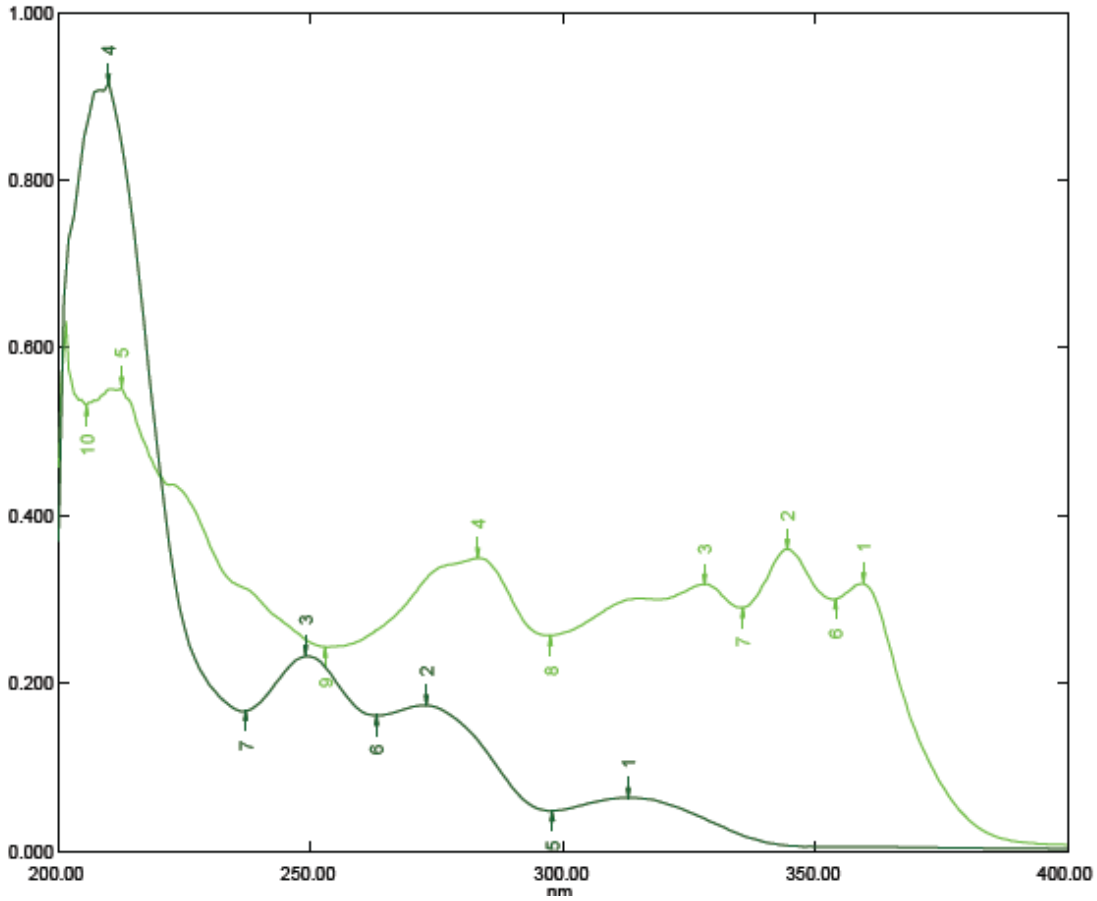


Fig: 7.1.1: Overlain Normal spectra of Acebrophylline and Montelukastin ethanol

7.1.2. VALIDATION OF THE METHOD

LINEARITY

Acebrophylline

Acebrophylline was found to be linear in a concentration range of 6-24µg/ml. The absorbances of these solutions were noted at wavelengths 250 and 273 nm, respectively. Calibration curves were plotted using concentration Vs absorbance at wavelength of 250nm and the slope, intercept and correlation coefficient values were found to be 0.023,0.045, and 0.998 respectively, fig. 7.1.2. At wavelength 273nm, slope, intercept and correlation coefficient values were found to be 0.017,0.038 and 0.997 respectively, fig. 7.1.3.

Fig: 7.1.2: Calibration graph of Acebrophylline at 250nm

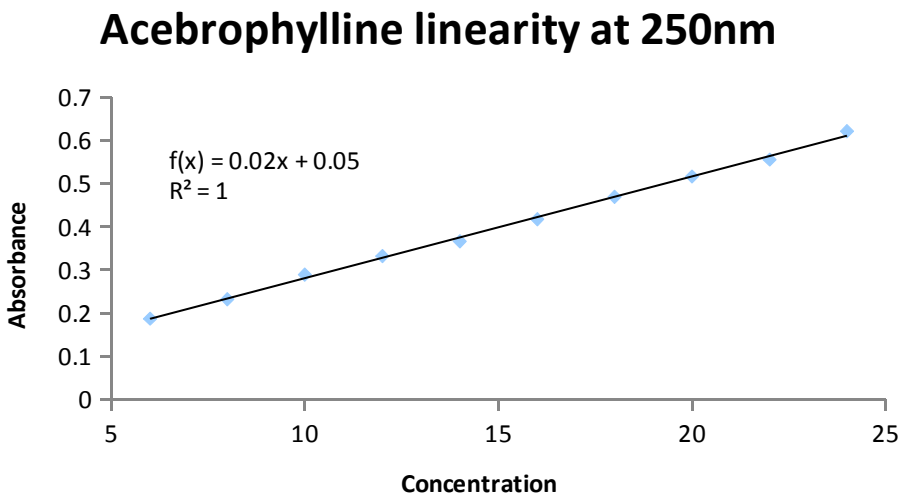


Fig: 7.1.3: Calibration graph of Acebrophylline at 273nm

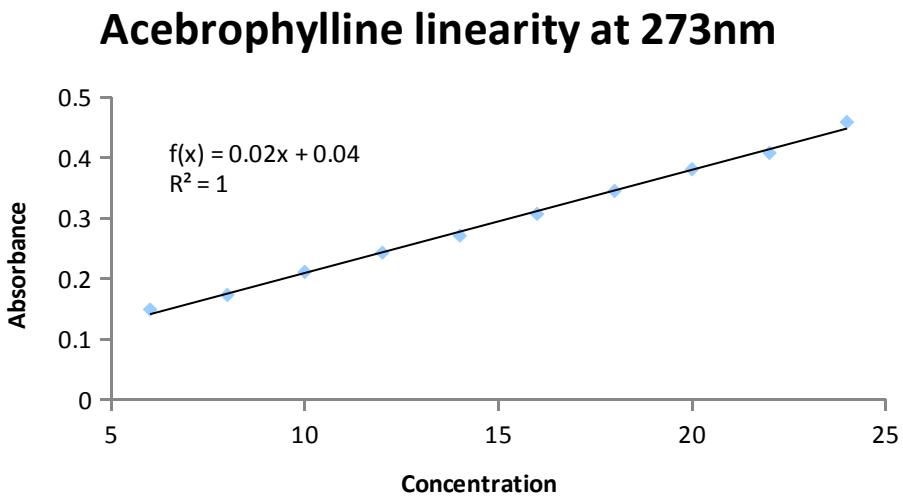


Table: 7.1.1. Calibration data of Acebrophylline at 250nm and273nm

S. No	Concentration (¬g/ml)	Absorbance at 250 nm	Absorbance at 273 nm
1	6	0.187	0.149
2	8	0.232	0.173

3	10	0.289	0.211
4	12	0.332	0.243
5	14	0.366	0.271
6	16	0.417	0.307
7	18	0.469	0.345
8	20	0.516	0.381
9	22	0.555	0.408
10	24	0.621	0.459

MontelukastSodium:

Montelukast was found to be linear at a concentration range of 6-24µg/ml. The absorbances of these solutions were noted at 250 and 273 nm, respectively. Calibration curves were plotted using concentration Vs absorbance. At a wavelength of 250nm, the slope, intercept and correlation coefficient values were found to be 0.032, 0.007 and 0.997 respectively, fig. 7.1.4.At wavelength 273 nm, the slope, intercept and correlation coefficient values were found to be 0.040, 0.018and 0.997respectively,fig. 7.1.5.

Fig: 7.1.4: Calibration graph of Montelukast at 250nm

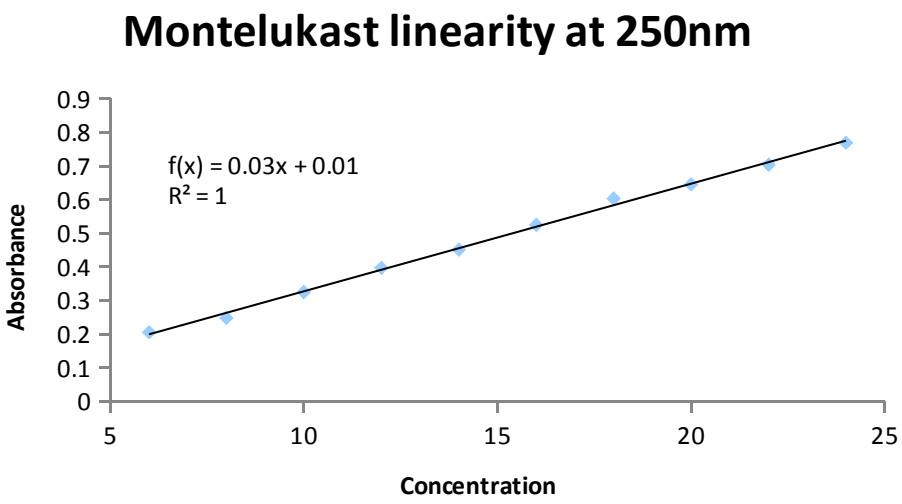


Fig: 7.1.5: Calibration graph of Montelukast at 273nm

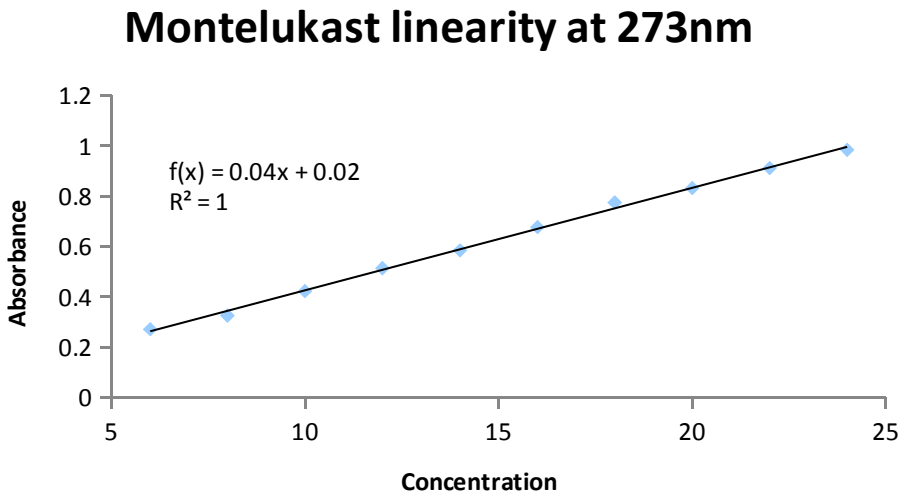


Table: 7.1.2. Calibration data of Montelukast at 250 and 273nm

S. No	Concentration (µg/ml)	Absorbance at 250 nm	Absorbance at 273nm
1	6	0.205	0.271
2	8	0.248	0.325
3	10	0.325	0.422
4	12	0.397	0.513
5	14	0.451	0.584
6	16	0.525	0.676
7	18	0.603	0.774
8	20	0.645	0.831
9	22	0.704	0.910
10	24	0.769	0.983

PRECISION

Precision studies were performed by preparing the standards three times and measuring the absorbances of drugs at 250 nm and 273 nm. Low RSD values indicate that the method is precise. The values are shown in table 7.1.3 and 7.1.4.

Table: 7.1.3. Intraday Precision studies

concentration	Absorbances				%RSD*			
	Acebrophylline		Montelukast		Acebrophylline		Montelukast	
	273 nm	250 nm	273 nm	250 nm	273 nm	250 nm	273 Nm	250 nm
10µg/ml	0.289	0.211	0.433	0.328	0.86	1.24	0.23	0.17
	0.292	0.215	0.434	0.329				
	0.294	0.216	0.432	0.328				
12µg/ml	0.332	0.243	0.515	0.389	0.46	0.82	0.11	0.14
	0.334	0.245	0.515	0.388				
	0.335	0.247	0.516	0.389				

Table: 7.1.4. Inter day Precision studies

concentration	Absorbance				%RSD*			
	Acebrophylline		Montelukast		Acebrophylline		Montelukast	
	273 nm	250 nm	273 Nm	250 nm	273 nm	250 nm	273 Nm	250 nm
10µg/ml	0.289	0.211	0.433	0.328	1.07	0.96	0.35	1.41
	0.285	0.209	0.430	0.328				
	0.283	0.207	0.432	0.320				
12µg/ml	0.332	0.243	1.877	0.059	0.76	0.83	1.01	0.91
	0.330	0.241	1.881	0.057				
	0.327	0.239	1.889	0.053				

Recovery studies

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. To an equivalent quantity of formulation powder (10mg), 9.5mg of standard Montelukast sodium was added to it (standard addition method), so that the sample contains 10mg each of Acebrophylline and Montelukast Sodium . The % recovery and %RSD were calculated as shown below.

Table: 7.1.5. Recovery studies

Level	%Recovery		%RSD	
	Acebrophylline	Montelukast	Acebrophylline	Montelukast
50%	98.33	98.5	0.1	0.27
100%	98.41	99.25	0.39	0.23

*RSD of three observations

7.1.3. ANALYSIS OF FORMULATION

Preparation of standard solutions

Standard stock solutions of Acebrophylline and Montelukast were prepared by dissolving 10 mg of the drug in ethanol and the volume was made up to 100ml in a standard flask. From the stock solution, concentrations ranging from 6-24µg/ml was prepared for both Acebrophylline and Montelukast and scanned in the UV region. An overlain spectrum of different concentrations of Acebrophylline and Montelukast and mixture are shown in fig. 7.1.6-7.1.7.

Preparation of sample solution

Sample stock solution:

Twenty tablets (ABROFYL-M) each containing 10 mg of montelukast sodium and 200mg of Acebrophylline were accurately weighed and the average weight was calculated and finely powdered. A quantity equivalent to 9.5 mg of Montelukast sodium was weighed and dissolved in ethanol in 100ml volumetric flask (standard addition

method).Sonicated to dissolve it completely and make volume up to the mark with ethanol. Mixed well and filtered through 0.45µm filter to get the final concentration of 100µg/ml.Finally 20mcg/ml working concentration of each of Acebrophylline and montelukast was prepared. Absorbances were noted at 250 nm and 273nm, respectively, fig. 7.1.8 and table 7.1.6. The amounts of Acebrophyllineand Montelukast were calculated using the **simultaneous equation** given below

$$\text{At } \mu_1 \; A_1 = ax_1 bc_x + ay_1 bc_y$$

$$\lambda_2 \; A_2 = ax_2 bc_x + ay_2 bc_y$$

$$C_{fvx} = \frac{A_2 ay_1 - A_1 ay_2}{ax_2 ay_1 - ax_1 ay_2}$$

$$C_{ofx} = \frac{A_1 ax_2 - A_2 ax_1}{ax_2 ay_1 - ax_1 ay_2}$$

A_1 = absorbance of formulation at 250nm.

A_2 = absorbance of formulation at 273nm.

ax_1 = Absorptivity of Acebrophylline at 250nm.

ax_2 = Absorptivity of Acebrophylline at 273nm.

ay_1 = Absorptivity of Montelukast at 250nm.

ay_2 = Absorptivity of Montelukast at 273nm.

C_{fvx} = Concentration of Acebrophylline.

C_{ofx} = Concentration of Montelukast.

Table: 7.1.6. Analysis of formulation

Drug	Amount (mg/tab)		% label claim	% RSD*
	Labelled	Found		
Acebrophylline	200mg	199.8	99.90	0.53
Montelukast	10mg	10.2	102	1.37

*RSD of three observations

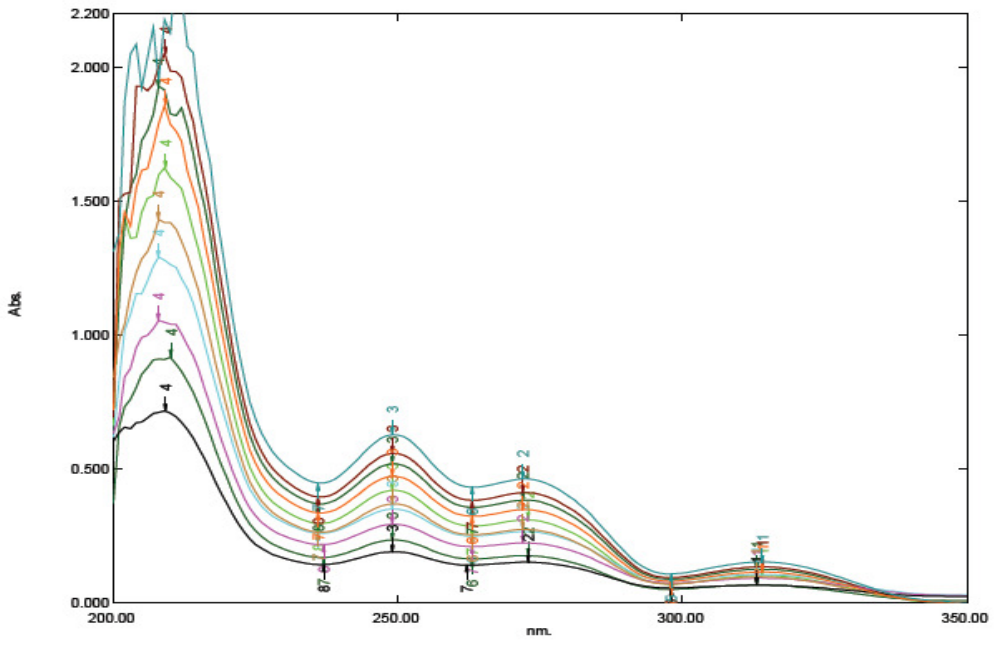


Fig: 7.1.6. Overlain UV spectra of Acebrophylline

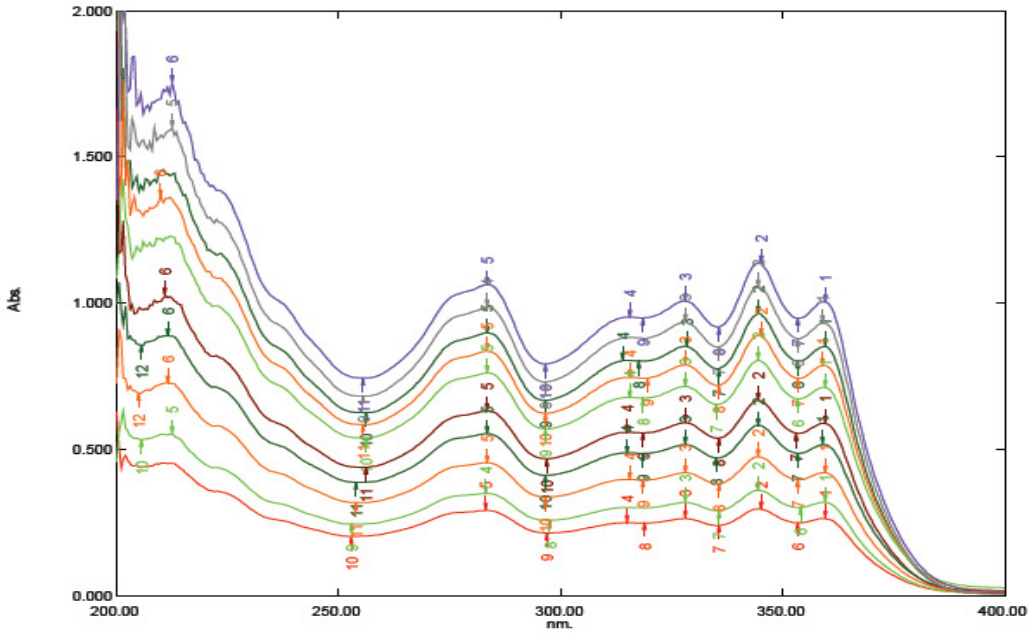
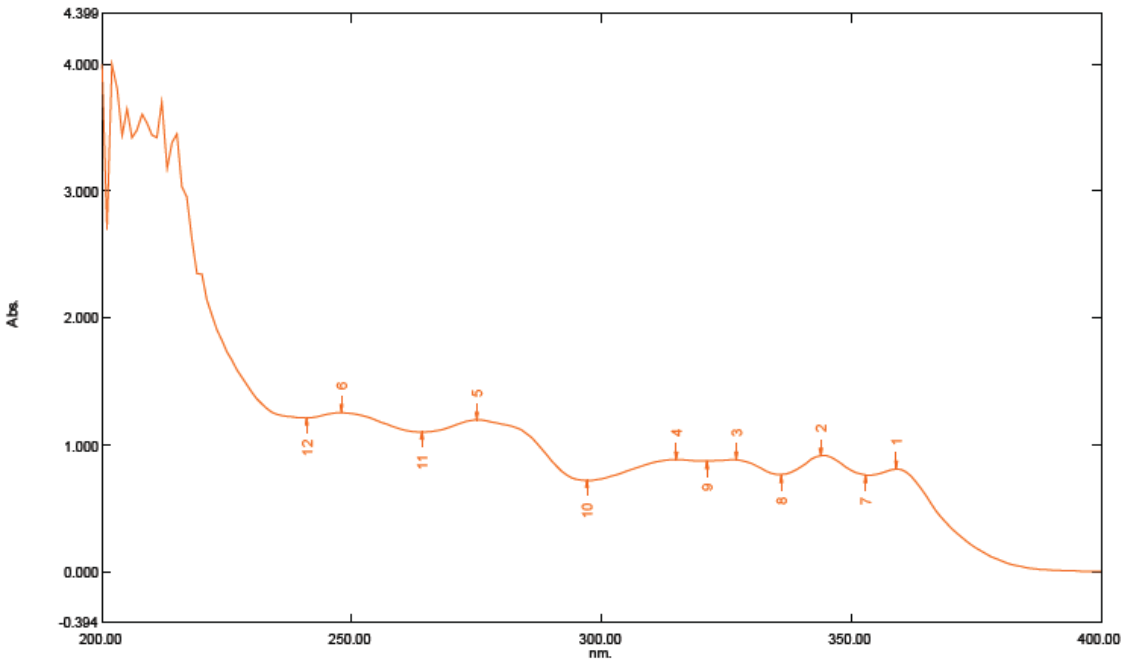


Fig: 7.1.7. Overlain UV spectra of Montelukast Sodium

Fig: 7.1.8. UV spectra of formulation



(20µg/ml of Acebrophylline and 20µg/ml of Montelukast)

7.2 DEVELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ACEBROPHYLLINE AND MONTELUKAST IN BULK ANDPHARMACEUTICAL DOSAGE FORM.

7.2.1. Selection of chromatographic method for separation

Reverse phase chromatographic technique is selected since both drugs are polar in nature.

7.2.2. Selection of wavelength

Sensitivity of HPLC method that uses ultraviolet (UV) detector depends upon the proper selection of wavelength. An ideal wavelength is the one that gives maximum absorbance and good response for the drug detected at lower concentration also.

From the UV spectra obtained for both drugs, 250nm was selected as the wavelength for study, fig. 7.2.1.

7.2.3. Initial chromatographic conditions:

Selection of Mobile phase:

For developing RP – HPLC method, different mobile phase systems with different ratios were tried, among which 10mM phosphate buffer and Acetonitrile (pH adjusted to 4 using 1% Orthophosphoric acid) gave symmetrical peaks with good resolution (Montelukast R_f – 6.7 minutes, Acebrophylline R_f – 3.6 minutes), and hence fixed for further studies.

Different mobile phases were tried and their observations are given below in the table.

Selection of Mobile Phase

S.no	Mobile phase conditions	Observation

1.	Water : Acetonitrile (30:70, v/v)	Tailing for Montelukast and Acebrophylline peak
2.	10 mMphosphate buffer: acetonitrile pH 8(30:70,v/v)	Splitting of Montelukast and Acebrophylline peak
3.	10 mM Phosphate buffer: acetonitrile pH 6(30:70,v/v)	Good, symmetrical peaks and less resolution.
4.	10 mM Phosphate buffer: acetonitrile pH 4 (85:15,v/v)	Good, symmetrical peaks and good resolution.

Chromatograms are shown in fig. 7.2.2-7.2.5.

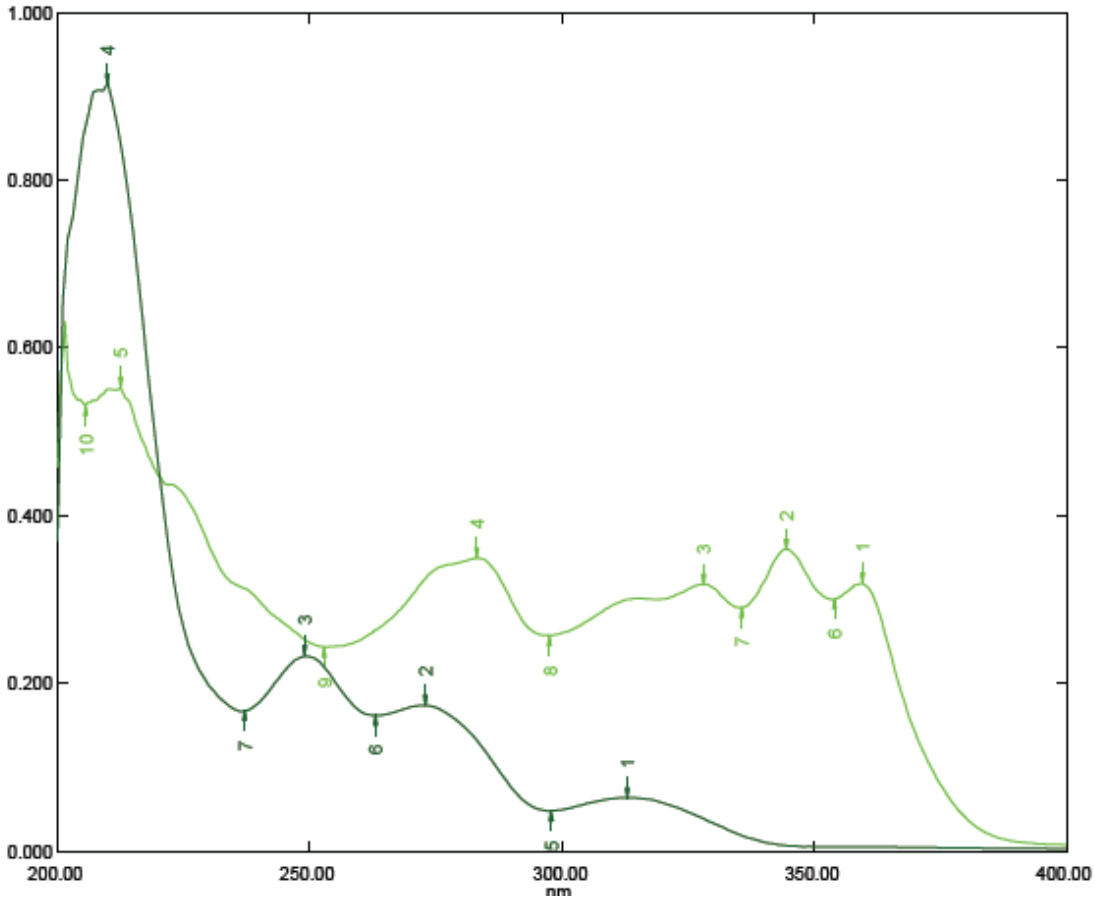


Fig: 7.2.1. UV spectra of Acebrophylline and Montelukast.

Selection of mobile phase

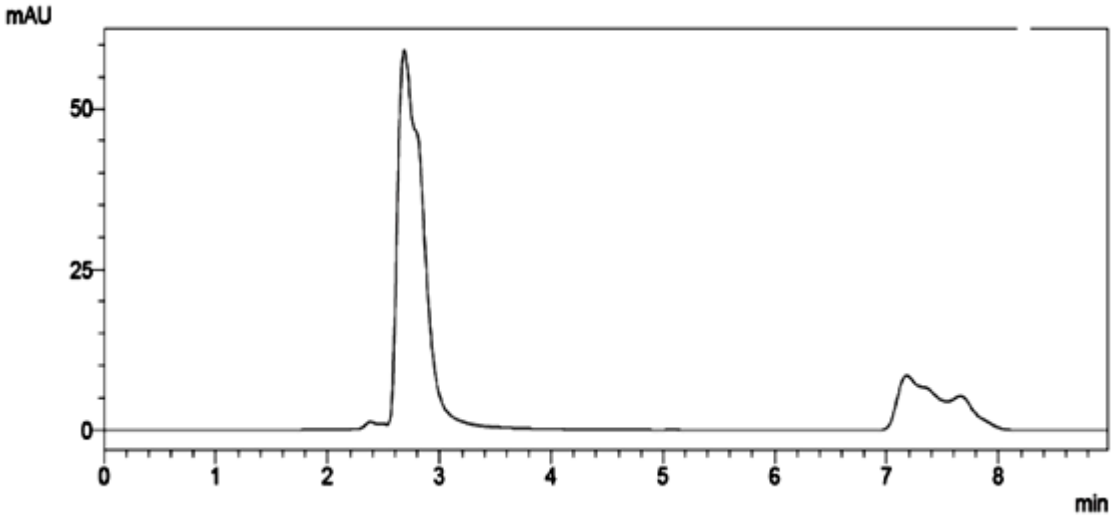


Fig: 7.2.2. Water and Acetonitrile (85:15)

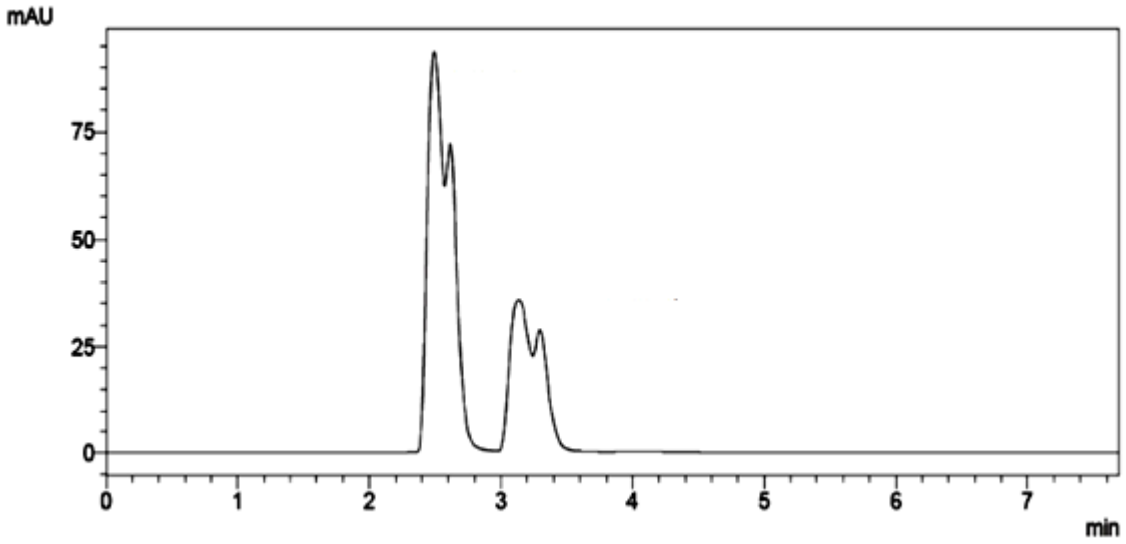


Fig:

7.2.3. 10mM phosphate buffer: Acetonitrile (85:15,v/v) pH 8

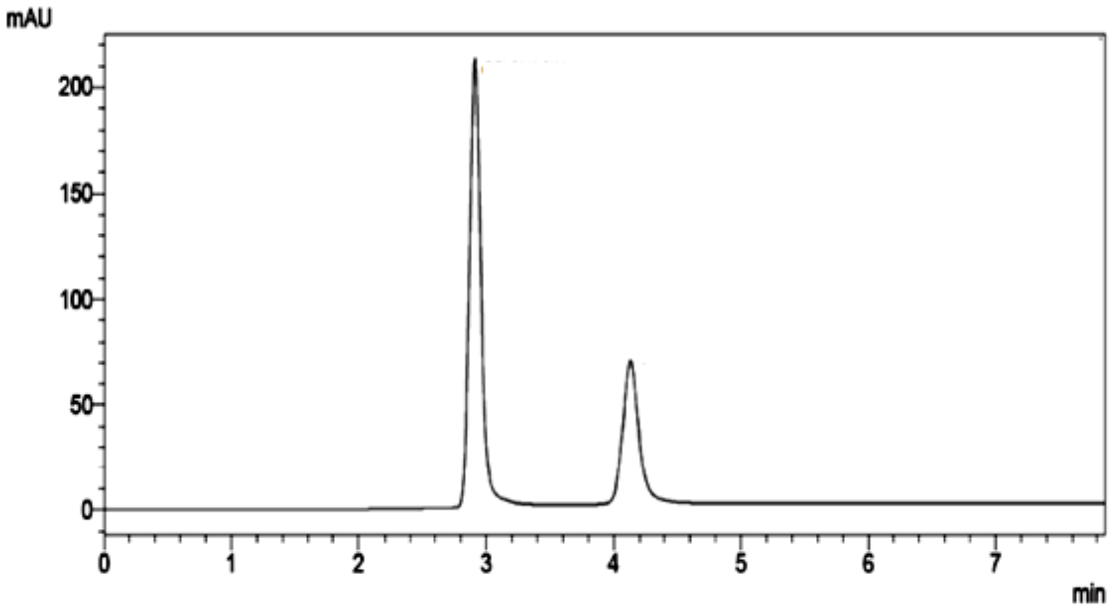


Fig:

7.2.4. 10mM phosphate buffer: Acetonitrile (85:15,v/v), pH 6

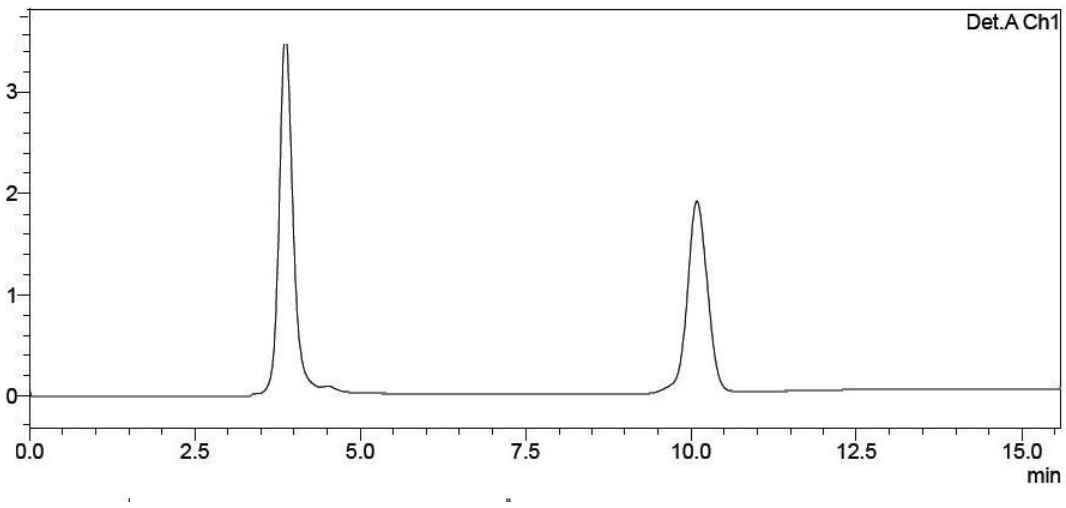


Fig: 7.2.5. 10mM phosphate buffer: Acetonitrile (85:15,v/v), pH 4

7.2.4. OPTIMIZATION OF SEPARATION CONDITION

Selection of wavelength

Different wavelengths ie., 273nm, 250nm, 315nm were taken into consideration from the UV spectras obtained. The wavelength250nm was finally selected as the impurities were less intensified and the peak intensities were good.

Effect of ratio of mobile phase

The mobile phase system consisting of phosphate buffer and acetonitrile in different ratios such as 10:90, 30:70, 85:15 % v/v, adjusted to pH 4 using 1% orthophosphoric acid were tried and the chromatograms were recorded at 250 nm at the flow rate of 1 ml/min (fig. 7.2.9-7.2.10). The ratio of 85:15 % v/v, gave good separation and symmetrical peaks and hence the ratio of 85:15% v/v was selected for the study.

Ratio (% v/v)	Retention time	
10mM phosphate:ACN	**Acebrophylline**	**Montelukast**
10:90	4.08	10.2
15:85	3.6	6.7
30:70	3.0	2.8

Effect of pH

Keeping other conditions constant, chromatograms were recorded with different pH such as 3,4,5,6 etc, adjusted using 1% orthophosphoric acid, fig. 7.2.11-

7.2.12. At the pH of 4, the peak shapes of both drugs were good and hence selected for further study.

pH	Observation
3	Tailing of peaks
4	No tailing, Symmetrical peaks
5	Split peaks
6	Broad Split peaks

Effect of strength of Phosphate buffer

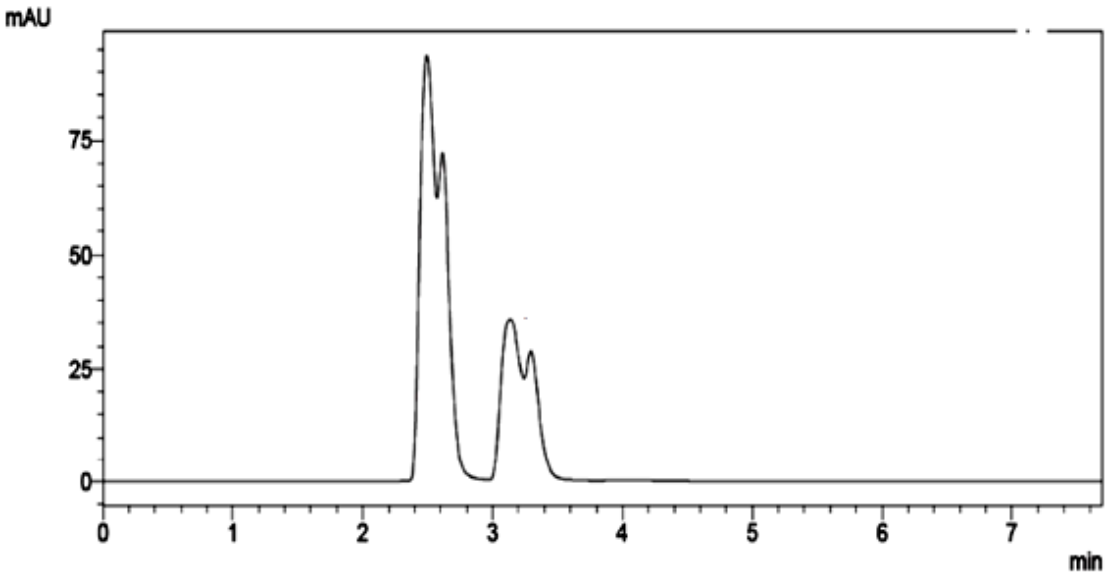


Fig: 7.2.6. 30mM Phosphate buffer: Acetonitrile (15:85)

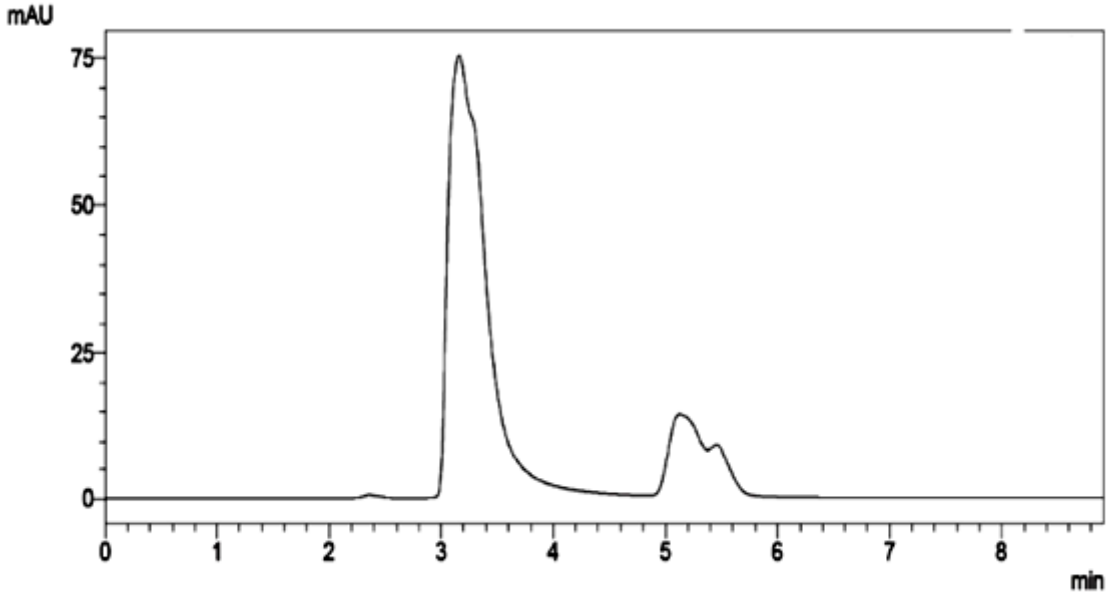


Fig: 7.2.7. 20mM Phosphate buffer: Acetonitrile (15:85)

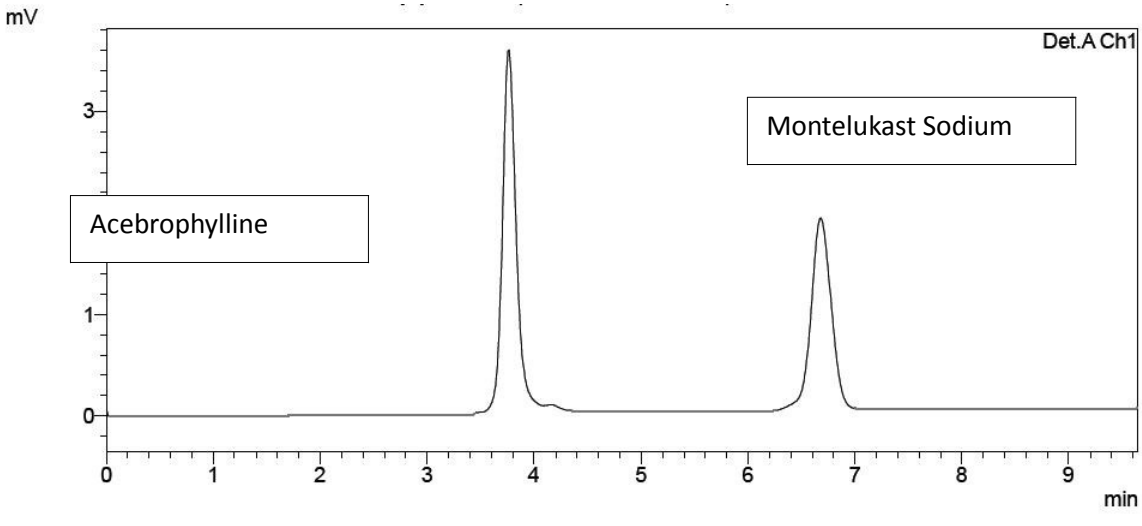


Fig:

7.2.8.10mM Phosphate buffer: Acetonitrile (15:85)

Effect of ratio of mobile phase

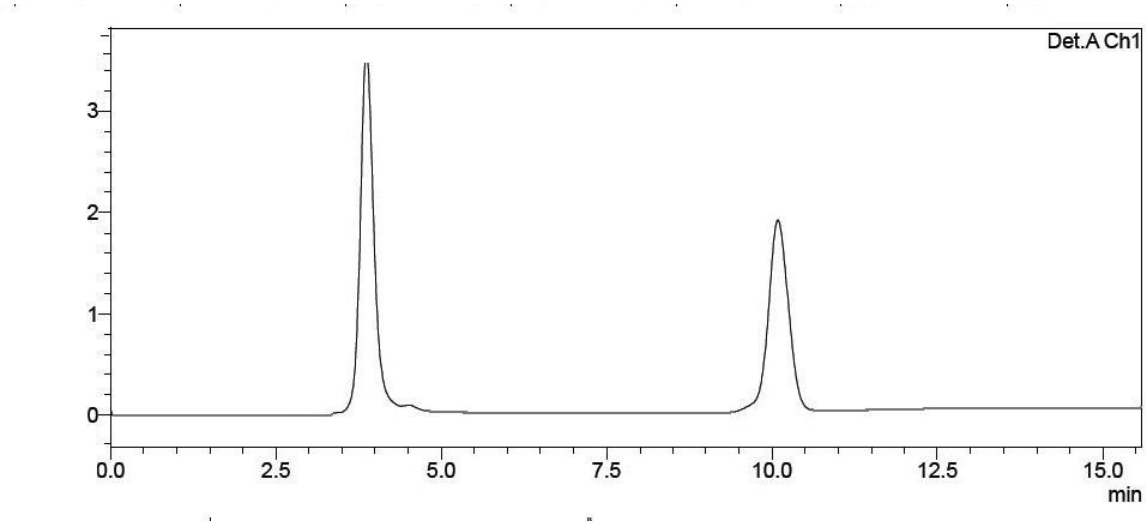


Fig: 7.2.9.10mMPhosphate buffer: Acetonitrile (10:90)

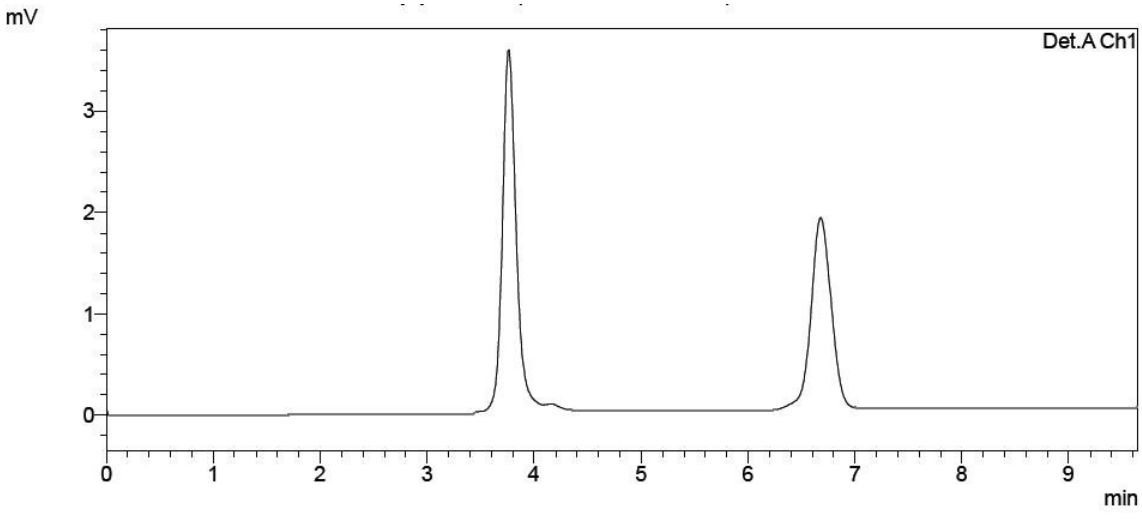


Fig: 7.2.10.

10mM Phosphate buffer: Acetonitrile (15:85)

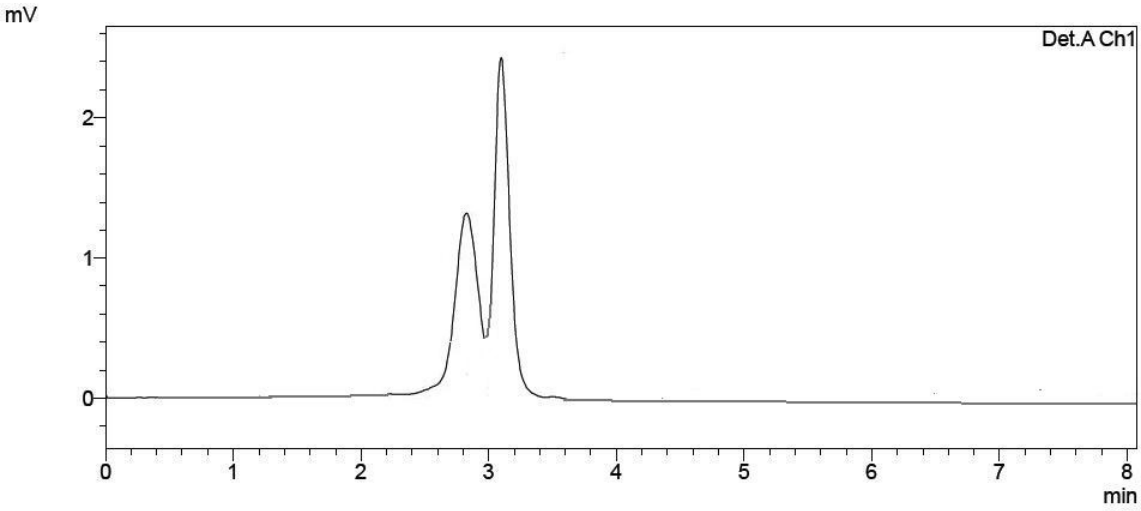


Fig: 7.2.11.10mM Phosphate buffer: Acetonitrile (30:70)

Effect of pH

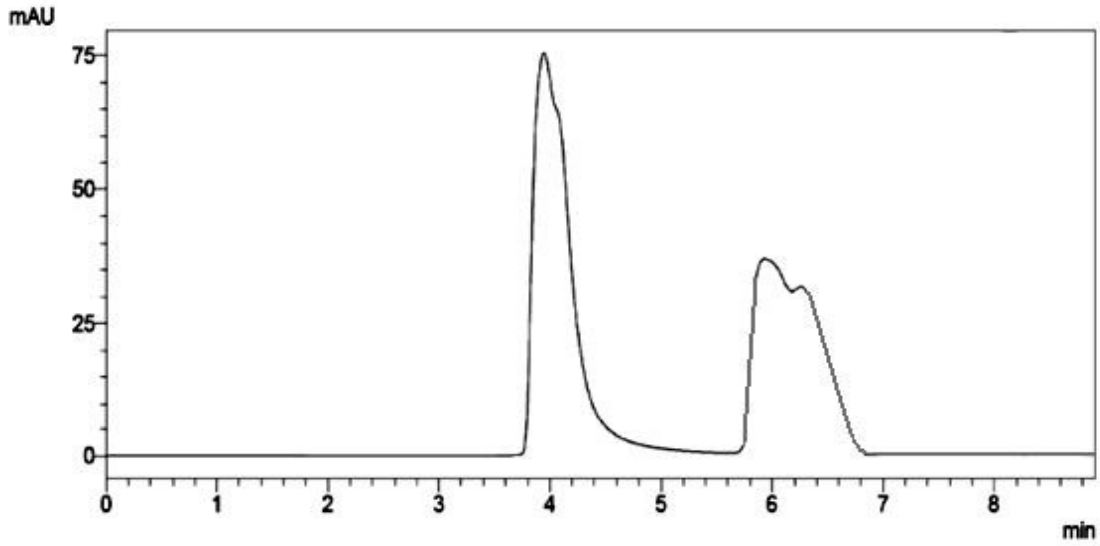


Fig: 7.2.12. 10 mM Phosphate buffer (pH 6): Acetonitrile (85:15)

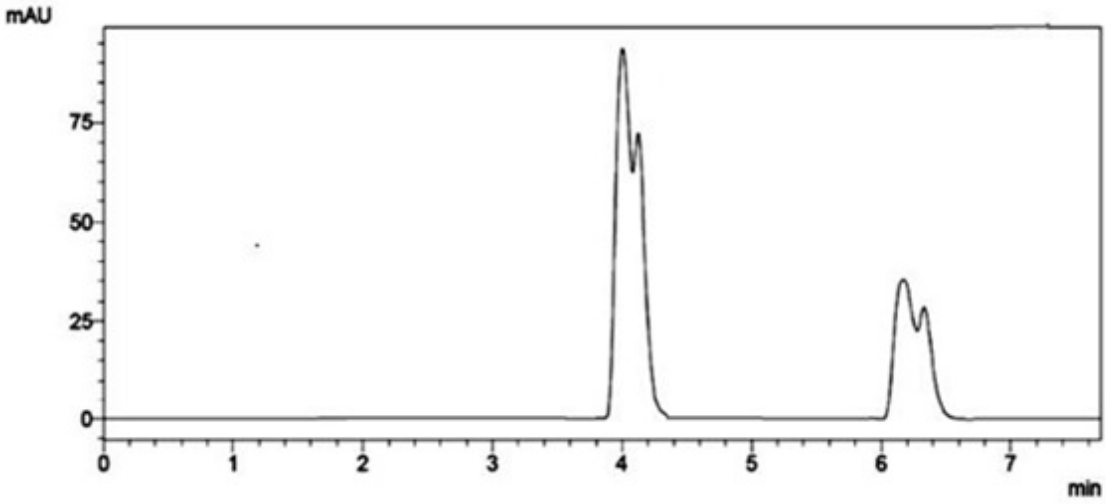


Fig: 7.2.13. 10 mM Phosphate buffer (pH 5): Acetonitrile (85:15)

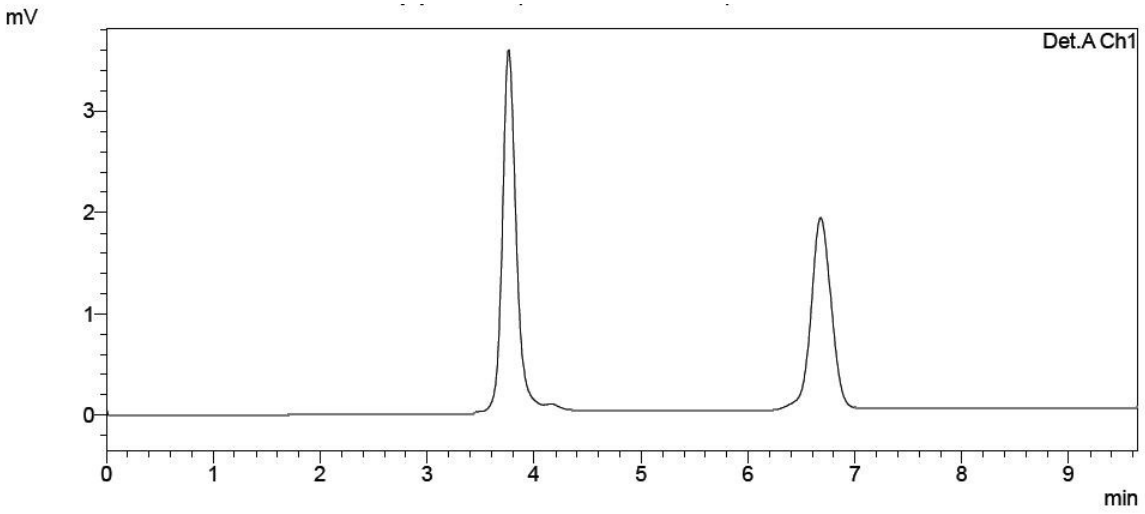


Fig: 7.2.14. 10 mM Phosphate buffer (pH 4): Acetonitrile (85:15)

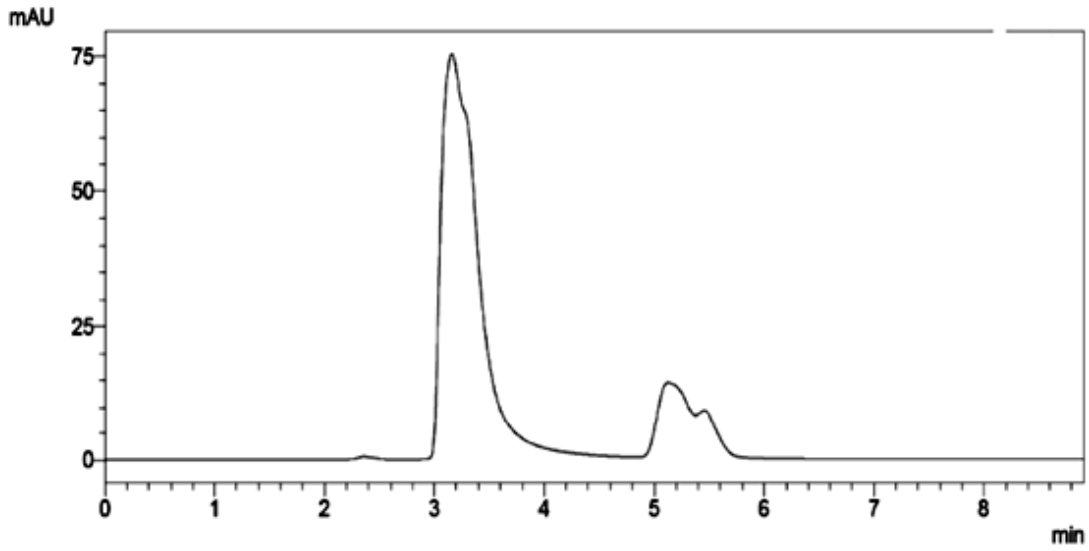


Fig: 7.2.15. 10 mM Phosphate buffer (pH 3): Acetonitrile (85:15)

7.2.5. FIXED CHROMATOGRAPHIC CONDITIONS

Stationary Phase	BDS Hypersil, C18 Column, (250 x 4.6mm , 5µ)
Mobile phase	10 mMphosphate buffer (pH 4): Acetonitrile
Solvent ratio	15:85
Detection wavelength	250 nm
Flow rate	1.0 ml/ml
Operating Pressure	60 kgf
Operating Temperature	Room Temperature
Injection volume	20µl
Run time	10 min
Mode of operation	Isocratic elution

7.2.6. VALIDATION OF RP – HPLC METHOD

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ of Acebrophylline and Montelukast were calculated mathematically. The LOD of Acebrophylline and Montelukast was found to be 0.087µg/ml and 0.010µg/ml. The LOQ of Acebrophylline and Montelukast was found to be 0.289µg/ml and 0.033µg/ml respectively

Linearity and Range

Acebrophylline and Montelukast were found to be linear in the range of 100-1000

ng/ml. Calibration graphs were plotted using peak areas of standard drugs vs. concentration of standard solutions, fig. 7.2.21-7.2.30. The slope, intercept and correlation coefficient values were found to be 41.64, 6219.9 and 0.997 respectively, for Acebrophylline and 29.821,8562 and 0.998 respectively, for Montelukast.

Precision

Precision of method was demonstrated by

- Intraday precision
- Inter day precision

a. Intraday precision

Intraday precision was found out by carrying out analysis of standard drug solutions at two different concentrations in the linearity range for three times on the same day and % RSD was calculated

Table: 7.2.1. Intraday Precision

Level	Concentration (ng/ml)		Peak Area		%RSD	
	ABR	MONTE	ABR	MONTE	ABR	MONTE
1	900	900	43087	35464	1.31	1.69
			44225	36062		
			43715	36685		
2	400	400	23276	20636	1.66	1.59
			24058	20825		
			23625	21281		

* RSD of three Observations

ABR- Acebrophylline, **MONTE**- Montelukast

b. Inter day precision

Inter day precision was found out by carrying out analysis of standard drug solutions at two different concentrations in the linearity range for three days over a period of one week and % RSD was calculated.

Table: 7.2.2. Inter day precision

Level	Concentration (ng/ml)		Peak Area		%RSD	
	ABR	MONTE	ABR	MONTE	ABR	MONTE
1	900	900	43087	35464	1.19	1.06
2			43562	35986		
3			44125	36212		
1	400	400	23276	20436	1.48	1.99
2			23598	20789		
3			23975	21265		

* RSD of three Observations

ABR- Acebrophylline, **MONTE**- Montelukast

Accuracy

To study the reliability, suitability and accuracy of the method, recovery studies were carried out by standard addition method. To an equivalent quantity of formulation powder (10mg), 9.5mg of standard Montelukast sodium was added to it (standard addition method), so that sample contains 10mg each of Acebrophylline and Montelukast sodium. Then a known quantity of standard Acebrophylline and Montelukast were added to 50% and 100% level extracted with mobile phase and made upto mark with same. This solution was further diluted. The concentration of drugs present in resulting solution was determined using assay method and percentage recovery and % RSD were calculated

Table:7.2.3.Recovery Studies

Drug	Amount added	Amount	% Recovery	% RSD*

	(mg) (%)	recovered (mg)		
MONTE	6(100%)	5.99	99.8	1.32
	3(50%)	3.00	100	1.26
ABR	6 (100%)	6.1	102	1.05
	3 (50%)	3.14	104	1.03

* RSD of three Observations

MONTE = Montelukast ,ABR = Acebrophylline

Robustness

1. In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

2. λ 2% in ratio of Acetonitrile in mobile phase, fig. 7.2.17-7.2.18.

3. ± 0.2 units in pH of buffer, fig. 7.2.19-7.2.20.

The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters and hence developed method is said to be robust. The values are shown in table 7.2.7

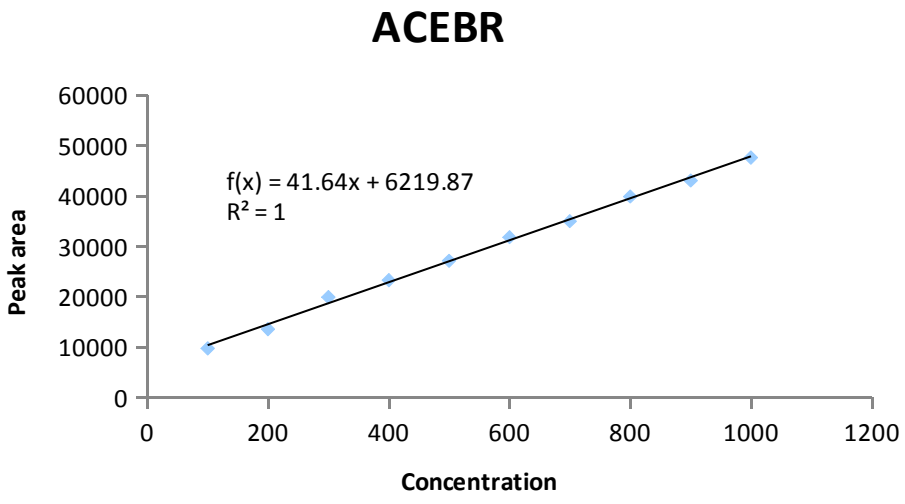


Fig: 7.2.16. Calibration graph of Acebrophylline by RP-HPLC.

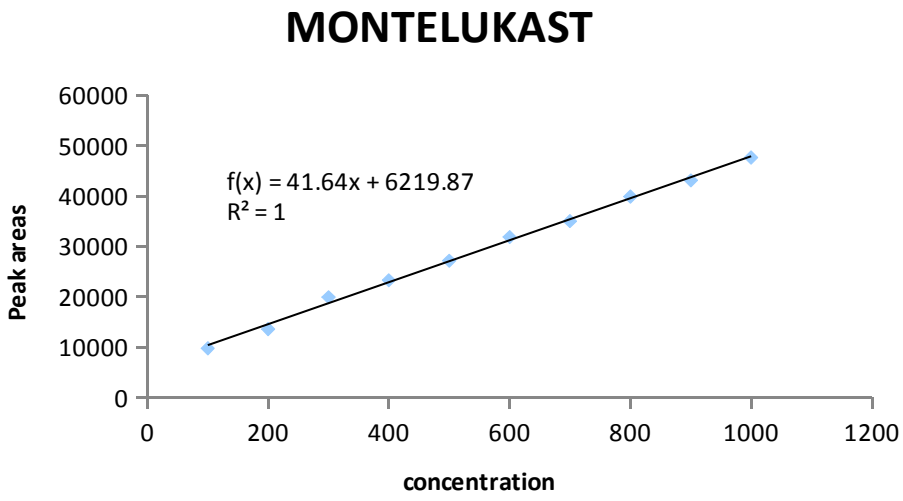


Fig: 7.2.17. Calibration Graph of Montelukast by RP-HPLC

Table: 7.2.4. Calibration data of Acebrophylline and Montelukast by RP-HPLC

S.No	Concentration (ng/ml)	Peak Areas	
		Montelukast	Acebrophylline
1	100	11573	9768
2	200	14880	13599
3	300	17168	19928

4	400	20436	23276
5	500	23738	27153
6	600	25771	31851
7	700	29963	35013
8	800	32119	39902
9	900	35464	43087
10	1000	38527	47642

Table: 7.2.5. Robustness data of Acebrophylline and Montelukast by RP-HPLC

Factor	Value	Peak Area		%RSD	
		MONTE	ABR	MONTE	ABR
pH	3.8 4 4.2	1264103 1256438 1238769	577442 589454 575789	1.0	1.2
Mobile Phase	10 mMPhosphatebuffer pH 4 and Acetonitrile in the ratio of (17:83, v/v)	1537653	625424		

	10 mMPhosphatebuffer pH 4 and Acetonitrile in the ratio of (15:85, v/v)	1558786	629746	1.3	0.4
	10 mMPhosphatebuffer pH 4 and Acetonitrile in the ratio of (13:87, v/v)	1579945	625087		

Robustness (change in mobile phase ratio)

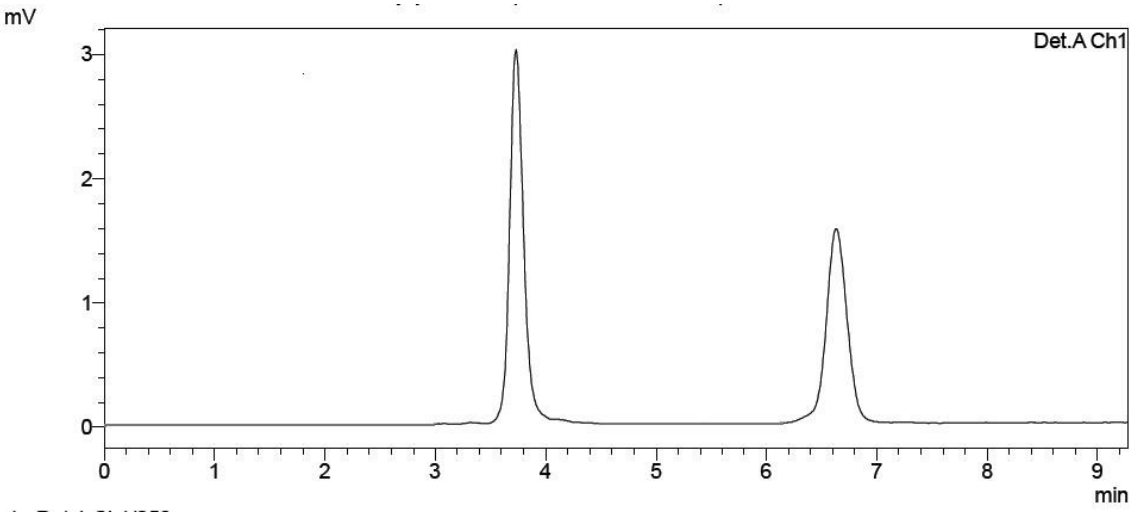


Fig: 7.2.18. 10 mM Phosphate buffer and Acetonitrile (17:83, v/v)

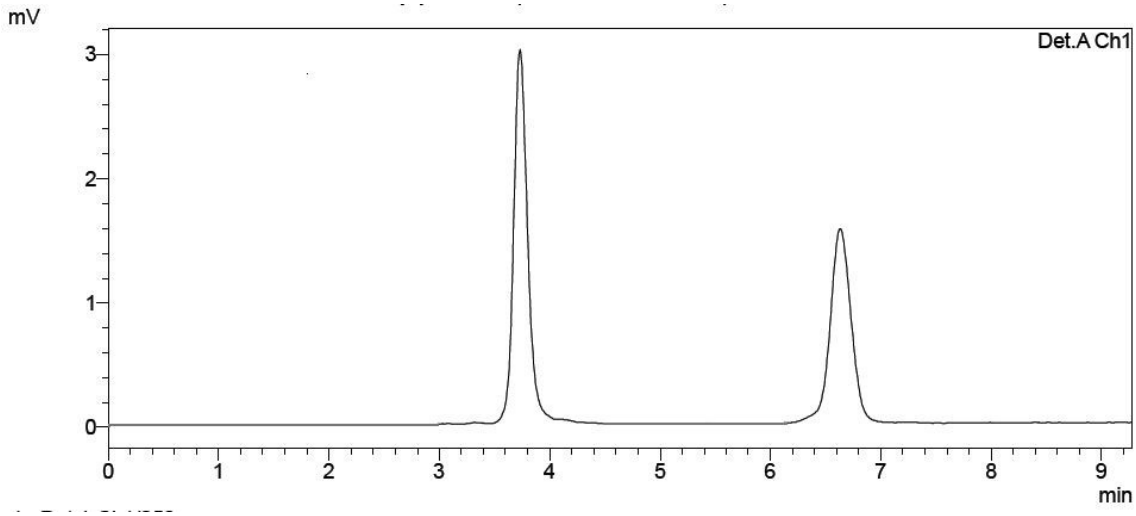


Fig: 7.2.19. 10 mM Phosphate buffer and Acetonitrile (13:87, v/v)

Robustness (change in the pH of the buffer)

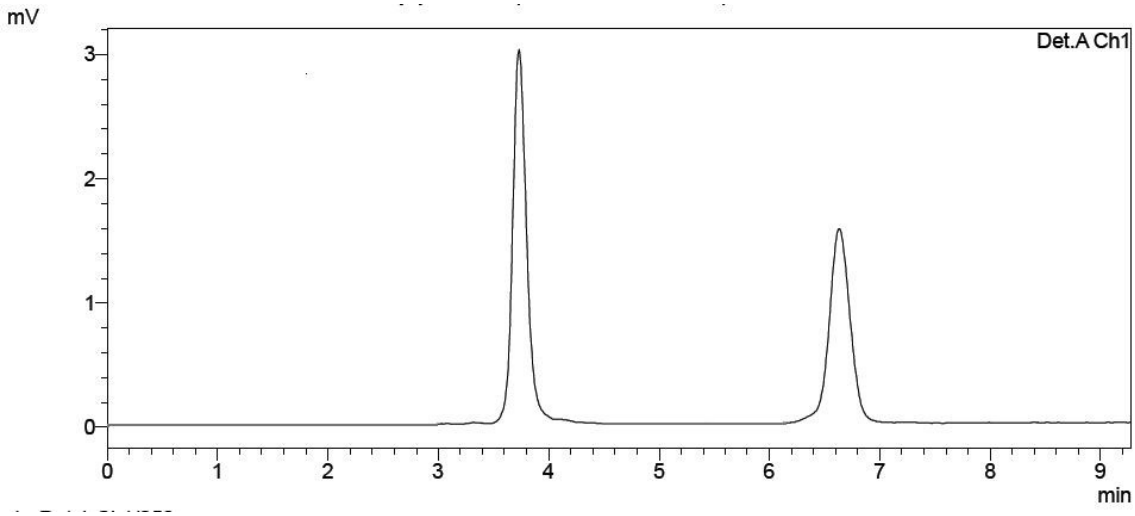


Fig: 7.2.20. 10 mM Phosphate buffer (pH 3.8): Acetonitrile (15:85)

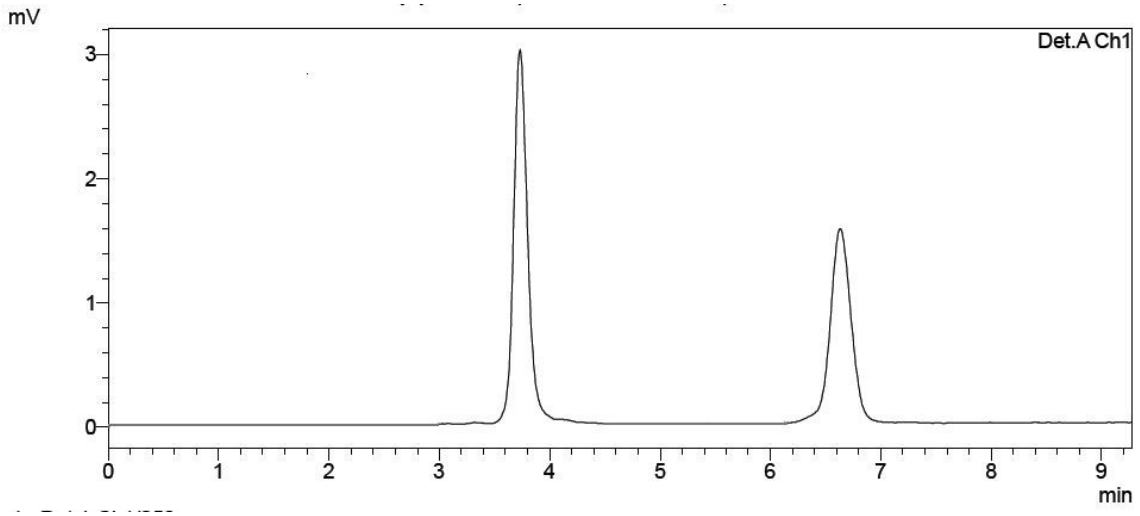


Fig: 7.2.21. 10 mM Phosphate buffer (pH 4.2): Acetonitrile (15:85)

7.2.7. ANALYSIS OF FORMULATION

Fixed Chromatographic conditions were applied for the analysis of formulation.

Preparation of Standard Solution

Stock solutions containing concentrations of 10µg/ml of Acebrophylline and Montelukast were prepared using mobile phase. This solution was suitably diluted to get aliquots of standard solutions containing 100 to 1000ng/ml of Acebrophylline and Montelukast.

Preparation of Sample Solution

Twenty tablets (ABROFYL-M) are powdered and the average weight was calculated. A quantity equivalent to 10 mg of drug was dissolved in mobile phase and to it known quantity of standard Montelukast were added. Finally the volume was made up to get a working concentration of 600 ng/ml each ofAcebrophylline and Montelukast.

Recording of Chromatograms

A steady baseline was recorded with the fixed chromatographic conditions. Standard drug solutions containing 100 to 1000ng/ml of Acebrophylline and Montelukast were injected and chromatograms were recorded, fig. 7.2.21-7.2.30.

Retention times of Acebrophylline and Montelukast were found to be 3.6 and 6.7minutes. This was followed by injection of sample solution obtained from the formulation, fig. 7.2.31

Calibration curves were plotted using peak areas of standard drugs vs concentration of corresponding standard solutions. Peak areas of the sample chromatograms were compared and amount of Acebrophylline and Montelukast were calculated and tabulated.

Table: 7.2.6.Analysis of Formulation

Drug	Label claim (mg)	Amount found (mg)	%Label claim	%RSD*
Montelukast	10	10.2	102	1.37
		10.4	104	
Acebrophylline	200	199.6	99.8	0.14
		200	100	

* RSD of three observations.

7.2.9. SYSTEM SUITABILITY TESTING

System suitability test parameters like Resolution, Retention Time, Theoretical plate and Tailing factor are shown in Table 7.2.7.

Table: 7.2.7. System suitability parameters

Parameter	Montelukast	Acebrophylline
Retention time (min)	6.6	3.7
Theoretical plate	4246	2568
Tailing factor	1.05	1.26
Resolution (min)	2.9	

CHROMATOGRAMS OF STANDARD SOLUTIONS

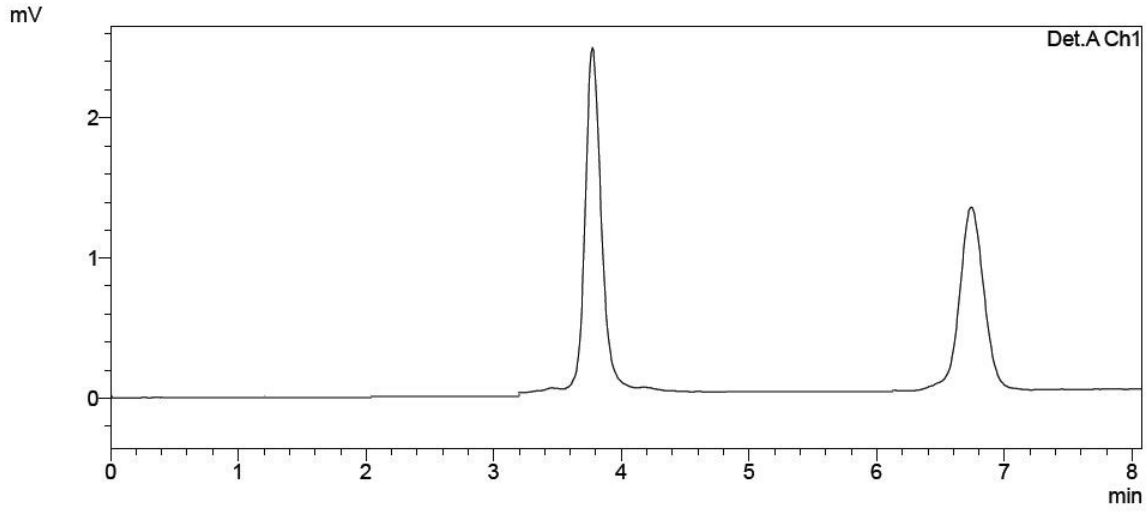


Fig:

7.2.22. 100 ng/ml of Montelukast and of Acebrophylline

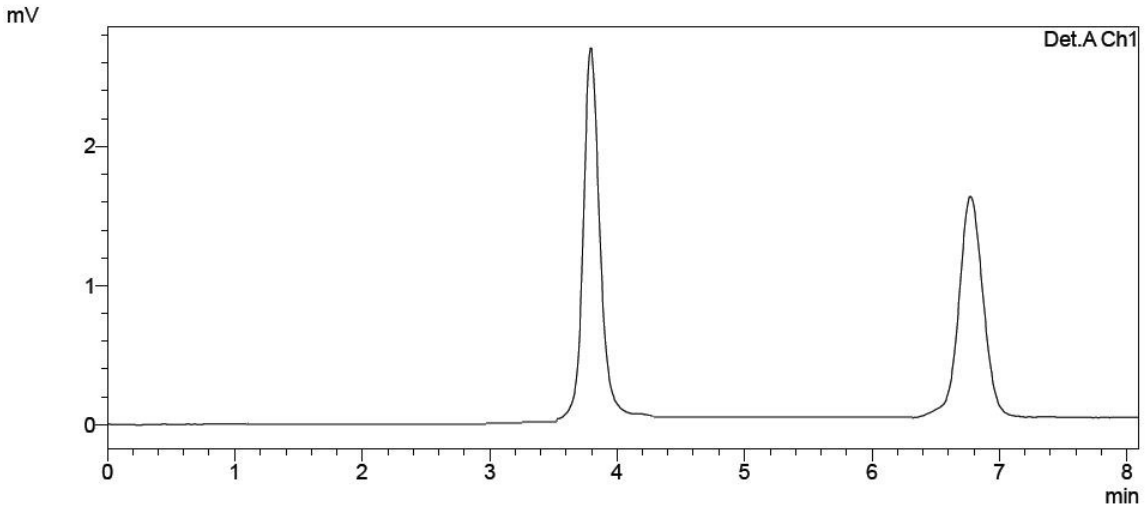


Fig:

7.2.23. 200ng/ml of Montelukast and Acebrophylline

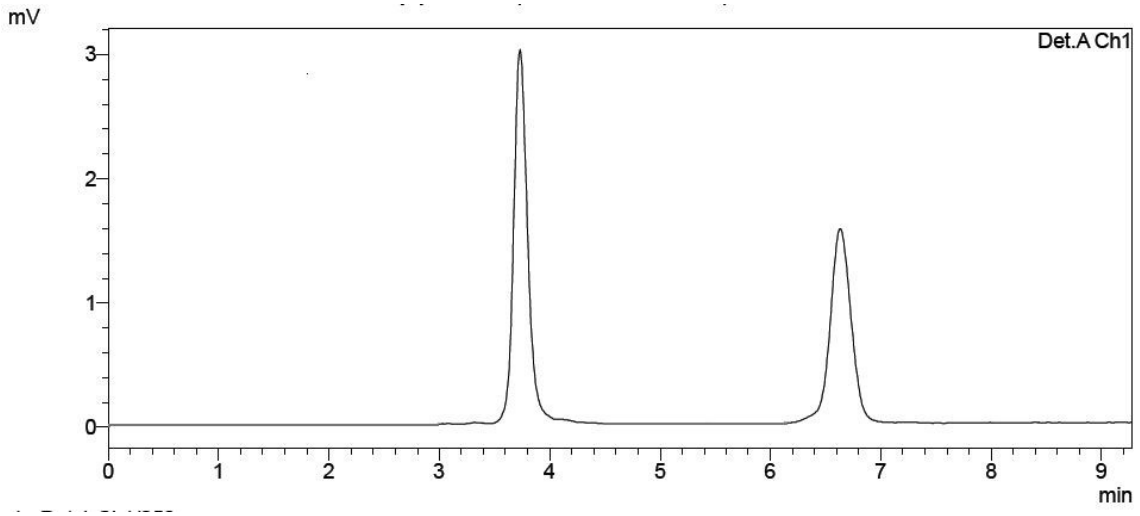


Fig: 7.2.24. 300 ng/ml of Montelukast and Acebrophylline

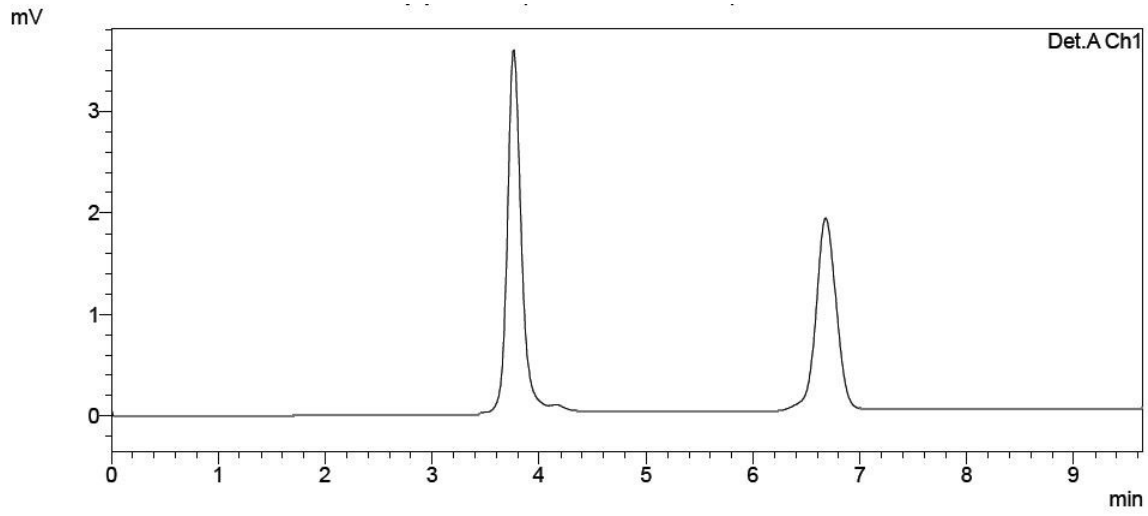


Fig: 7.2.25. 400ng/ml of Montelukast and Acebrophylline

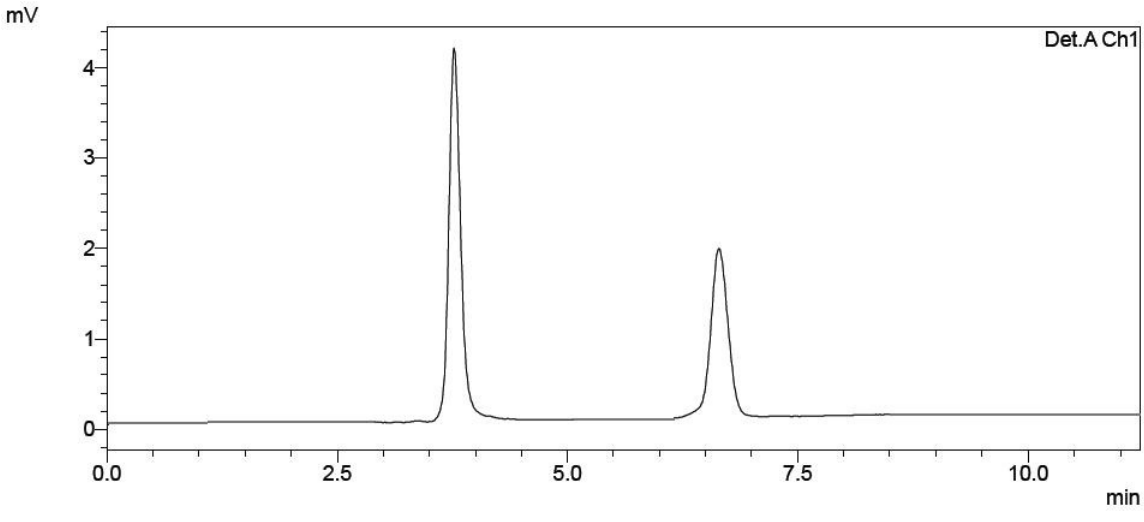


Fig: 7.2.26. 500ng/ml of Montelukast and Acebrophylline

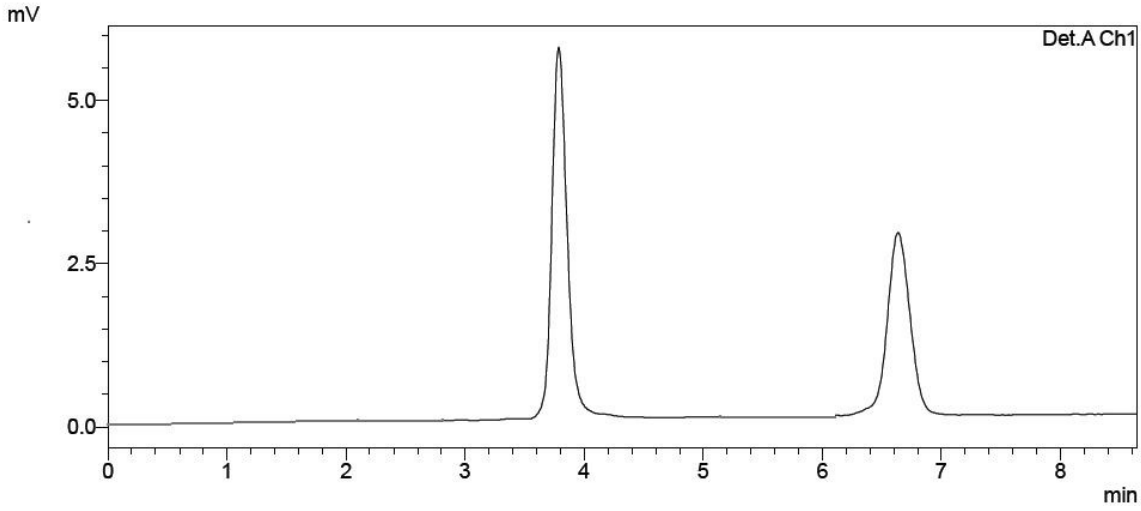


Fig: 7.2.27. 600ng/ml of Montelukast and Acebrophylline

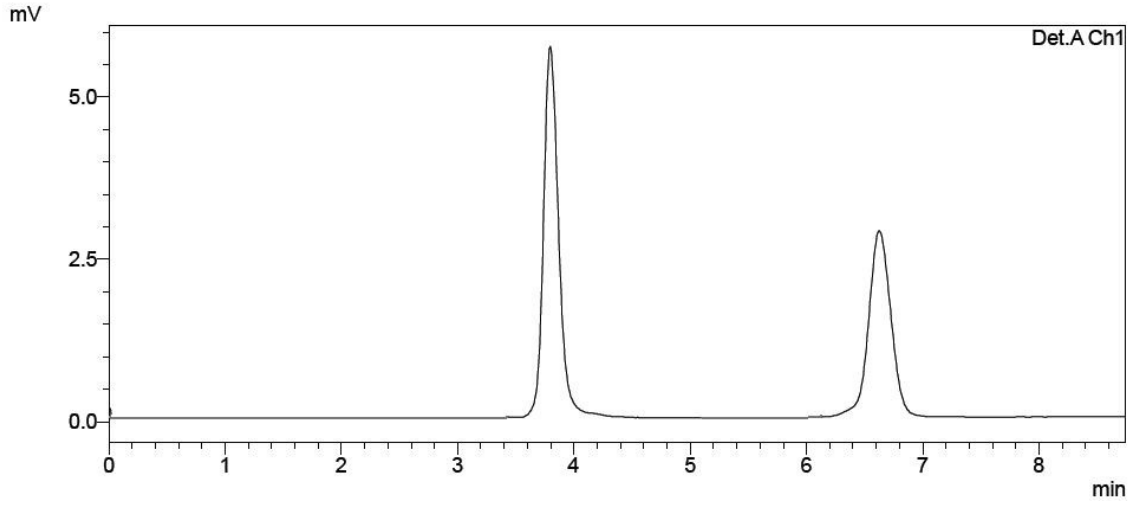


Fig: 7.2.28. 700 ng/ml of Montelukast and Acebrophylline

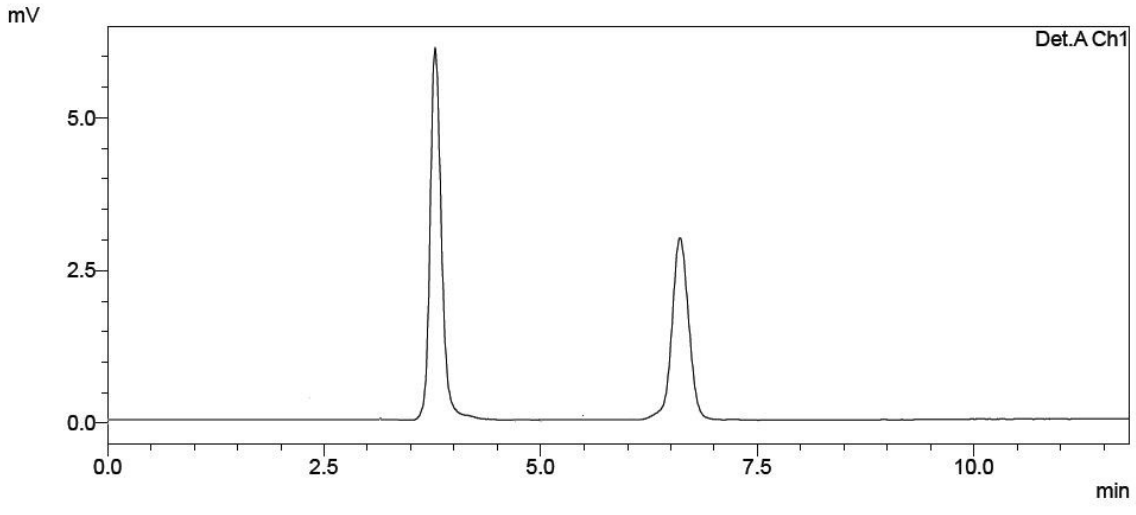


Fig:

7.2.29. 800 ng/ml of Montelukast and Acebrophylline

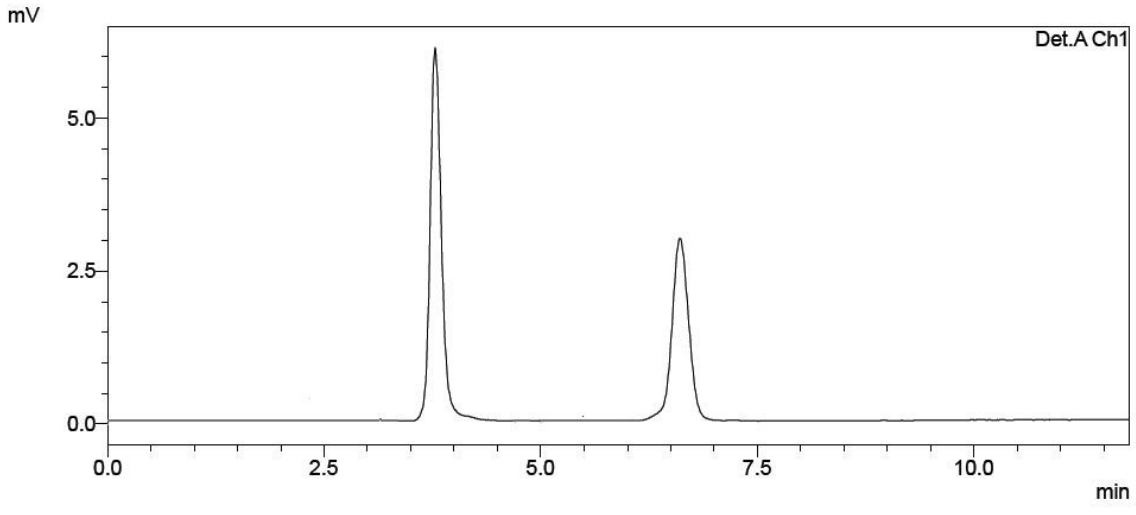


Fig:

7.2.30. 900 ng/ml of Montelukast and Acebrophylline

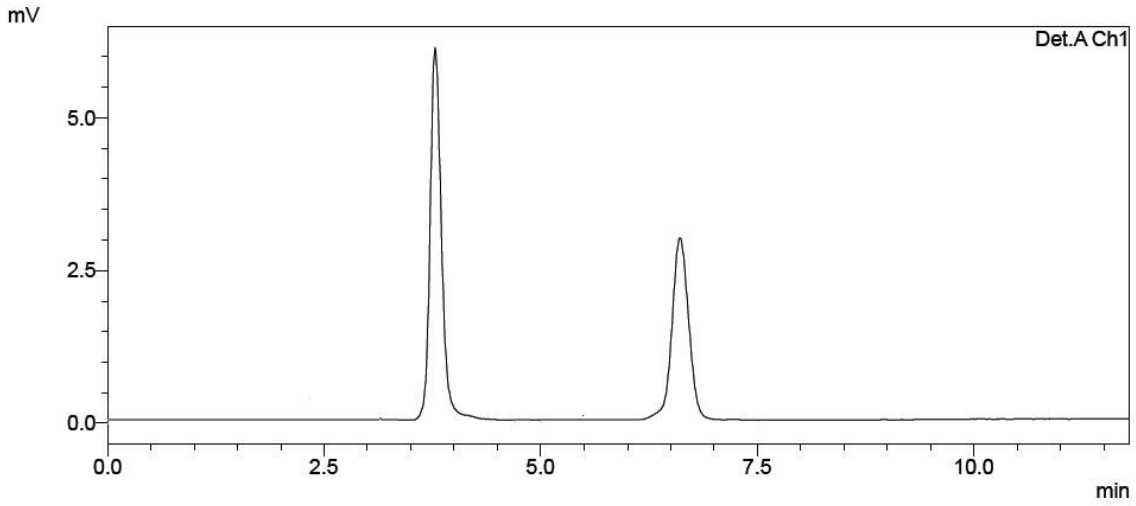


Fig: 7.2.31. 1000ng/ml of Montelukast and Acebrophylline

Analysis of formulation

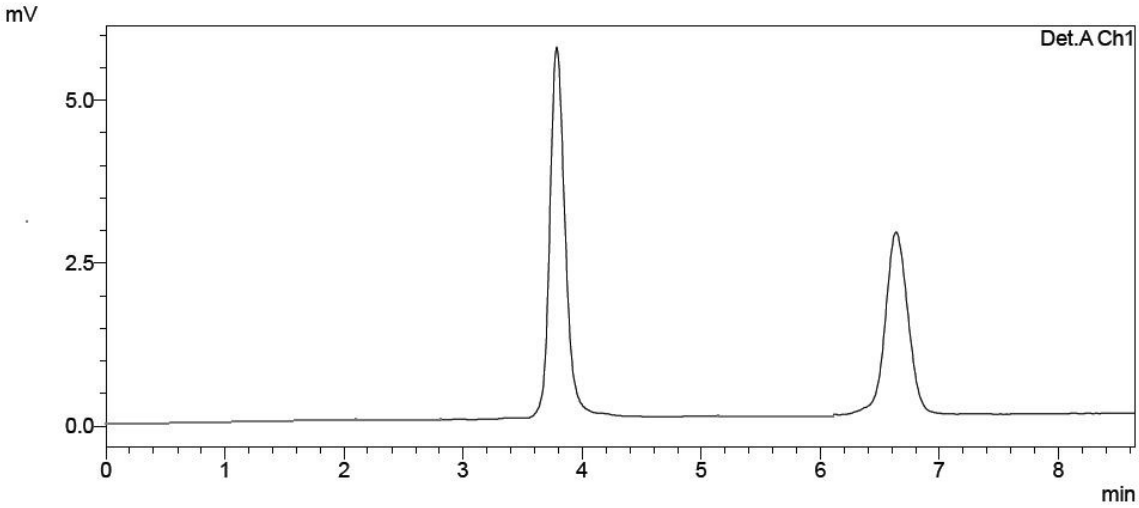


Fig: 7.2.32. 600ng/ml of Montelukast and Acebrophylline

8.RESULTS AND DISCUSSION

8.1. UV SPECTOSCOPIC METHOD

8.1.1. Simultaneous equation method

Estimation of Acebrophylline and Montelukast sodium was achieved by simultaneous equation method using 1800 double beam UV-Visible spectrophotometer. The linearity was checked in different concentrations and Beers law obeyed in the concentration range of 6 to 24µg/ml for both Acebrophylline and Montelukast sodium. The slope, intercept and correlation coefficient values of Acebrophylline and Montelukast sodium at 250nm are 0.023, 0.045, 0.998 and 0.032, 0.007, 0.997. The slope, intercept and correlation coefficient values of Acebrophylline and Montelukast sodiumat 273nm are 0.017, 0.038, 0.997 and0.040, 0.018 and 0.997.

The recovery studies were carried out to ensure the reproducibility and reliability of the method by adding known amount of standard drugs and analysis was carried out as per formulation procedure.

8.2. HPLC METHOD

In this method, optimization of different chromatographic parameters

like selection of

- Chromatographic method for separation
- Detection wavelength
- Different ionic strengths of mobile phase
- Mobile phase ratio
- Mobile phase pH
- Flow rate etc., were done.

A binary mixture of phosphate buffer and Acetonitrile was selected as the initial mobile phase system for the determination of both drugs. A wavelength of 250nm was selected for present study. Firstly, various concentrations of Phosphate buffer were

tried. From this, 10mM phosphate buffer was found to be ideal for the work. Then different pH's of buffer were tired, out of which Phosphate buffer adjusted to pH 4 with Orthophosphoric acid gave good peaks. Then the ratio of mobile phase was determined by varying the proportion of Phosphate buffer and Acetonitrile. Finally, the mixture of 10mM phosphate bufferbuffer adjusted to pH 4 with Orthophosphoric acid and Acetonitrile (15:85% v/v) was employed for the simultaneous determination of both drugs. The retention times of Acebrophylline and Montelukast sodium were found to be 3.7 and 6.6minutes respectively.

The developed method was validated as per ICH guidelines. Calibration graphs were potted using standard peak areas vs. concentration of standard solutions. The slope, intercept and correlation coefficient values were found to be 29.821, 8562.5 and 0.998 for Montelukast sodium and 41.64, 6219.9, 0.997 for Acebrophylline respectively. Acebrophylline and Montelukast sodium were found to be linear in the range of 100 to 1000ng/ml. The LOD of Acebrophylline and Montelukast sodium were found to be 0.087µg/ml and 0.010µg/ml respectively. The LOQ of Acebrophylline and Montelukast sodium were found to be 0.289µg/ml and 0.033µg/ml respectively. Precision of the developed method was studied under intraday precision and interday precision of the injection. Low % RSD values indicate that the method is precise. The developed method was found to be robust. The validated liquid chromatographic method was applied to simultaneous determination of Acebrophylline and Montelukast.

9. SUMMARY AND CONCLUSION

SUMMARY

- Optimization of the mobile phase was performed based on resolution, asymmetric factor and peak area obtained for both Acebrophylline and Montelukast sodium.
- The mobile phase combination of 10mM Phosphate buffer: acetonitrile 15:85PH 4.00 (adjusted with Orthophosphoric acid) found to be satisfactory and gave two symmetric and well resolved peaks for Acebrophylline and Montelukast sodium.
- The retention time for Acebrophylline and Montelukast sodium were 3.7 and 6.6, respectively.
- The method gives good resolution between both the compounds with a short analysis time
- The method was validated as per ICH Guidelines.
- The calibration curve for Acebrophylline was obtained by plotting the peak area of Acebrophylline versus the concentrations of Acebrophylline and Montelukast over the range of 100-1000ng/ml , and it was found to be linear with r2 = 1. The recoveries of Acebrophylline and Montelukast sodium were found to be in the range of 98%-100% and 100%-104% within precision RSD of 1.32 and 1.05 for Acebrophylline and Montelukast sodium.
- The system suitability parameters such as theoretical plates and tailing factor were found to be 2568, 1.26 and 4246, 1.05 respectively for Acebrophylline and Montelukast sodium.
- The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method.
- The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The detection limit (LOD) was found to be 0.08µg/ml for Acebrophylline and 0.01µg/ml for Montelukast sodium respectively.

- The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10).
- The quantitation limit (LOQ) was found to be 0.289µg/ml for Acebrophylline and 0.033µg/ml for Montelukast sodium respectively.
- The method was found to be simple, sensitive, accurate and precise.
- Proposed study describes a new RP-HPLC method for estimation of Acebrophylline and Montelukast sodium combination in mixture using simple mobile phase.
- Therefore, the proposed method can be used for routine analysis of Acebrophylline and Montelukast sodium their combined dosage form.

CONCLUSION

In the current study a new RP-HPLC method for estimation of Acebrophylline and Montelukast sodium combination in mixture using simple mobile phase was developed, optimized and validated. The developed method is simple, sensitive, accurate and precise. The developed method can be used for routine analysis of Acebrophylline and Montelukast sodium in a combined dosage form.

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